Characterisation of the gut microbiota in three porcine models of obesity and metabolic syndrome

The impact of dietinduced obesity on the dynamic and profile of gut microbiota

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PhD thesis by

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2012

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Preface

This PhD thesis was performed in the Microbial Ecology group at National veterinary institute, Technical university of Denmark, Copenhagen at the department of Bacteriology, Pathology and Parasitology under supervision of Professor Mette Boye and co-supervisor Jan Stagsted. Duration of PhD project was from December 2008 till October 2012. The project was partly financed by funds provided to the DTU National Veterinary Institute and co-financed by a grant from the Danish Strategic Research Council (FØSU 2101-06-0034) which was part of the project: "Nutriomics – functional foods for cloned lean/obese pigs".

Three papers make the basis of this thesis and throughout the thesis the papers will be referred to by their roman number. Table 1 summarises the different studies in each paper.

Paper I: **Pedersen R**., Andersen AD., Mølbak L., Stagsted J., Boye M.: "Changes in the gut microbiota of cloned and non-cloned control pigs during development of obesity". 2012, *submitted to BMC Microbiology*

Paper II: **Pedersen R**., Andersen AD., Stagsted J., Boye M.: "The gut microbial composition of lean and obese cloned and non-cloned pigs on high-energy diet". 2012, s*ubmitted to Microbial Ecology*

Paper III: **Pedersen R.,** Ingerslev HC., Sturek M., Alloosh M., Cirera S., Berit Ø. Christoffersen BØ., Moesgaard SG., Larsen N., Boye M.: "Characterization of intestinal microbiota in Ossabaw minipigs and Göttingen minipigs as models of obesity and metabolic syndrome". 2012, *submitted to PlosOne*

Table 1 Summary of the studies performed in each paper

Study	Model	Diet	Phenotype	Sample	Method	Studied
Paper I	Cloned Domestic pigs	HF/HE-a*d libitum*	Obese	Faeces	T-RFLP qPCR(RotorGene)	- Bacterial diversity in the faecal microbiota during the diet intervention period - Time-line analysis: change in bacterial diversity
	Non-cloned Domestic pigs	HF/HE- *ad libitum*	Obese	Faeces	T-RFLP qPCR(RotorGene)	- *Firmicutes*/*Bacteroidetes* ratio-during diet intervention study - Correlation between body-weight and *Firmicutes*/*Bacteroidetes* Ratio
Paper II	Cloned Domestic pigs	HF/HE-restrictive (Lean) HF/HE-*ad libitum* (obese)	Lean and obese	Faeces, colon, terminal ileum content	T-RFLP qPCR (RotorGene) High throughput qPCR (Fluidigm)	- Bacterial diversity in the faecal microbiota of lean group during the diet intervention period - *Firmicutes*/*Bacteroidetes* ratio in lean and obese pigs in colon and terminal ileum microbiota
	Non-cloned Domestic pigs	HF/HE-restrictive (Lean) HF/HE-*ad libitum* (obese)	Lean and obese	Faeces, Colon, terminal ileum content	T-RFLP qPCR (RotorGene) High throughput qPCR (Fluidigm)	- Fold differences in different taxonomic groups between lean and obese pigs - Fold differences in different taxonomic groups between lean/obese cloned and non-cloned pigs
Paper III	Göttingen minipigs	Lean-minipig chow Obese-minipig chow *ad libitum* Lean-mini pig chow	Lean and obese	Colon, cecum content	Sequencing by Illumina High throughput qPCR	- Characterization of intestinal microbiota in Phyla, class, family and genus level - Fold differences in different taxonomic groups between lean and obese pigs - Göttingen minipigs: Differences between lean and obese intestinal microbiota in colon and cecum
	Ossabaw Minipigs	Lean: standard chow (2200 kcal/day) Obese: atherogenic diet (HF/HE) (4500-600 kcal/day)	Lean and obese (MetS)	Colon, terminal ileum content	Sequencing by Illumina High throughput qPCR	- Ossabaw minipigs: Differences between lean and obese intestinal microbiota in colon and terminal ileum

Summary

Obesity is increasing worldwide at an alarming rate which has reached epidemic proportions. Obesity and its co-morbidities such as cardiovascular disease, type-2 diabetes and cancer are of great economical burden to the countries affected. Efforts have been made to identify the causal factors that lead to obesity and maintenance of the obese state. Recently the gut microbiota has been implicated of being a contributing factor to obesity. Therefore the gut microbiota is a potential target for management of obesity and its co-associated morbidities by changing the composition of the microbiota by pre- and probiotics diet supplements.

In order to investigate the impact of diet and obesity on gut microbiota and vice versa in humans, there is a need for translational animal models that can contribute to the understanding of obesity and its related diseases. Pigs are often used in intervention studies, primarily due to the close resemblance of their anatomy and physiology to that of humans.

The main focus of this Ph.D. thesis was to elucidate the changes in gut microbiota during the course of diet induced obesity in three porcine models and to relate their microbial profiles to a physiological trait, namely lean or obese phenotype. Furthermore, these porcine models of obesity were evaluated for their potential as animal models that may be used in diet-induced obesity-gut micriobiota related studies in the future.

In this Ph.D. thesis, the faecal and intestinal microbiota was investigated in three different breeds of pigs that were used as models of obesity: the domestic Danish pig (Landrace x Yorkshire), Göttingen minipigs and Ossabaw minipigs. The L x Y pigs were divided into two groups; cloned pigs- and non-cloned pigs used as the control group for the cloned pigs.

The gut microbiota was primarily studied with molecular methods such as terminal restriction fragment length polymorphism (T-RFLP), fluorescent in situ hybridization (FISH), next generation sequencing by Illumina and two quantitative real-time PCR platforms namely the Rotor-Gene Q instrument and high-throughput microfluidics-dynamic array.

The faecal, colonic and ileal microbiota of lean and obese, cloned and non-cloned pigs was investigated by T-RFLP and the results showed that the gut microbiota was affected by high-fat/high-energy diet and obesity. A positive correlation was observed between body-weight and percentage of body-fat in obese cloned and in non-cloned pigs. Based on gut microbial profile, the composition of the microbiota in cloned pigs did not have less inter-individual variations among them as compared to the microbiota in non-cloned pigs. The bacterial diversity was similar between cloned and non-cloned pigs over time in both the lean and obese group. The colon microbiota of lean cloned pigs contained relatively more of bacteria belonging to the phylum *Firmicutes* and less bacteria belonging to the phylum *Bacteroidetes*

than obese cloned pigs. In the obese group, body-weight correlated positively with the relative abundance of *Firmicutes* and negatively with the abundance of *Bacteroidetes*; however the negative correlation with *Bacteridetes* was only observed in the cloned pigs.

According to the next generation sequencing data (the V5 region of the 16S rRNA gene) the microbiota of cecum from obese Göttingen minipigs was characterised by significantly higher abundances of *Spirochaetes*, *Tenericutes* and *Verrucomicrobia* at phyla level than the lean Göttingen minipigs' cecal microbiota. Generally, most of the differences in bacterial groups were observed in cecal microbiota. Based on qPCR, there was 7.6-fold more *Clostridium* cluster XIV in cecal microbiota of obese Göttingen minipigs as compared to lean Göttingen. In lean Göttingen minipigs' cecal microbiota, significantly higher abundances of the phyla *Firmicutes* and genera *Clostridium*, *Akkermensia* and *Methanovibribacter* was observed compared to obese Göttingen minipigs. Overall, most significant differences between lean and obese Göttingen minipigs cecal microbiota was observed in Gram-negative bacteria both at phyla and genera taxonomic levels.

The composition of the intestinal microbiota of lean and obese Ossabaw minipigs was investigated by sequencing as in the Göttingen study. The results revealed that the microbiota in colon and terminal ileum of obese Ossabaw minipigs had higher ratios of *Firmicutes* to *Bacteroidetes* than the lean Ossabaw minipigs. Furthermore, in obese Ossabaw minipigs, a significantly higher abundance of the genus *Clostridium* was observed in colon and terminal compared to lean Ossabaw minipigs. In lean Ossabaws the abundances of bacteria belonging to the genera *Prevotella* and *Lactobacillus* were significantly higher in both colon and terminal ileum than the obese Ossabaws. The high ratio of *Firmicutes* to *Bacteroidetes* has previously been connected to the obese phenotype in mice and humans. While low abundances of *Lactobacillus* in obesity has been reported to be connected to dysmetabolism in obese mice.

In conclusion, the cloned pigs did not have reduced inter-individual variation as compared to non-cloned pigs in regard to their gut microbiota, in neither the obese nor the lean state. The gut-microbial analysis of all the lean and obese domestic pigs suggests that high-fat/high-energy diet both in restricted amounts and *ad libitum* is associated with an increase in *Firmicutes* in the gut microbiota. Overall, high-fat/high-energy diet affects the gut microbiota differently than normal chow as observed in cloned/non-cloned L x Y pigs, Ossabaw and Göttingen minipigs, respectively.

Sammendrag

På verdensplan, er fedme stigende i et alarmerende tempo, der har nået epidemiske proportioner. Fedme og dens følgesygdomme såsom hjertekarsygdomme, type-2 diabetes og kræft er en enorm økonomiskbyrde for de berørte lande. Der er gjort bestræbelser på at identificere de kausale faktorer, der fører til fedme og vedligeholdelse af den fede tilstand. For nylig har forskning vist, at sammensætningen af tarmens mikrobiota kan være en medvirkende faktor til fedme. Derfor er tarmens mikrobiota et potentielt mål for modulering af fedme og dens co-associerede morbiditeter ved hjælp af diæt med tilsætning af præ-og probiotika.

For at undersøge virkningen af kost og fedme på tarmens mikrobiota og omvendt, er der behov for translationelle dyremodeller, der kan bidrage til forståelsen af fedme og dens følgesygdomme. Grise anvendes ofte i interventionsstudier, primært på grund af lighed af deres anatomi og fysiologi til mennesker.

Hovedfokus i denne Ph.d. afhandling var at belyse ændringerne i tarmens mikrobiota i løbet af kost-induceret fedme i tre grisemodeller og at relatere deres mikrobielle profiler til et fænotypisktræk, nemlig normalvægtige eller fede grise. Endvidere er disse grisemodeller blevet evalueret for deres potentiale som dyremodel, der kan anvendes i kost-induceret fedme-tarm mikrobiota relaterede undersøgelser i fremtiden.

I denne Ph.d. afhandling blev mikrobiotaen i fæces og forskellige steder i tarmen undersøgt i tre forskellige racer af grise, der blev brugt som dyremodeller for fedme: Danske produktionssvin (Landrace x Yorkshire), Göttingen minigrise og Ossabaw minigrise. L x Y svin blev inddelt i to grupper; klonede grise, og ikke-klonede grise der blev brugt som kontrolgruppe for de klonede grise.

Tarmens mikrobiota blev primært undersøgt med molekylære metoder, såsom terminal restriktion fragment længde polymorfisme (T-RFLP), fluorescerende *in situ* hybridisering (FISH), næste generation sekventering ved Illumina og to kvantitative real-time PCR metoder ved brug af Rotor-Gene Q instrument og high-throughput mikrofluid-dynamisk array (Fluidigm).

Sammensætningen af mikrobiotaen i fæces, kolon og ileum i tynde og fede, klonede og ikke-klonede grise blev undersøgt ved T-RFLP, og resultaterne viste, at tarmens mikrobiota var påvirket af diet med højt-fedt/høj-energi indhold og fedme. En positiv korrelation mellem kropsvægt og procent kropsfedt blev observeret hos overvægtige klonede og ikke-klonede grise. Baseret på tarmen mikrobielle profil, har de klonede grise ikke mindre variation i sammensætningen af deres tarm mikrobiota blandt dem end ikke-klonede grise. Den bakterielle diversitet var det samme mellem klonede og ikke-klonede grise over tid i både tynde og fede grise. Mikrobiotaen i kolon af de tynde klonede grise indeholdt relativt flere bakterier fra phylum *Firmicutes* og færre bakterier fra phylum *Bacteroidetes* end fede klonede

grise. I de fede klonede og ikke-klonede grise, korrelerede kropsvægt positivt med den relative forekomst af *Firmicutes* og negativt med *Bacteroidetes*, men den negative korrelation med *Bacteroidetes* blev kun observeret i de klonede grise.

Sammensætningen af mikrobiotaen i Göttingen minigrise blev undersøgt ved næste generations sekventering af V5-regionen af 16S rRNA-genet. De fede Göttingen minigrises' cecum mikrobiota var karakteriseret ved signifikant højere mængder af *Spirochaetes*, *Tenericutes* og *Verrucomicrobia* på phyla niveau end tynde Göttingen minigrise. Generelt blev de fleste forskelle i bakterielle grupper observeret i cecum mikrobiotaen. Baseret på qPCR analyser der var 7.6 fold mere *Clostridium* cluster XIV i cecum mikrobiotaen i de fede Göttingen minigrise end de tynde. Den cecum mikrobiota i slanke Göttingen minigrise havde signifikant højere mængde af phylum *Firmicutes og* slægterne *Clostridium*, *Akkermensia* og *Methanovibribacter* sammenlignet med fede Göttingen minigrise. Generelt blev de mest signifikante forskelle mellem tynde og fede Göttingen minigrises cecum mikrobiota observeret hos Gram-negative bakterier på såvel phyla som slægt taksonomiske niveauer.

Den intestinale mikrobiota af tynde og fede Ossabaw minigrise blev ligeledes undersøgt ved næste generations sekventering som i Göttingen undersøgelsen. Resultaterne viste, at den intestinale mikrobiota af de fede Ossabaw minigrise havde højere mængde af *Firmicutes* og lavere forekomst af *Bacteroidetes* i kolon og terminal ileum end de slanke Ossabaw minigrise. Endvidere fandtes en signifikant højere forekomst af slægten *Clostridium,* observeret i mikrobiota fra kolon og terminal ileum fra fede Ossabaw minigrise. I slanke Ossabaw minigrise var forekomsten af bakterier tilhørende slægterne *Prevotella* og *Lactobacillus* væsentligt højere i både kolon og terminal ileum end hos de fede Ossabaw minigrise. Den høje ratio af *Firmicutes* til *Bacteroidetes* er tidligere blevet knyttet til den fede fænotype. Mens mindre mængder af *Lactobacillus* er blevet rapporteret i fedme og er blevet forbundet til dysmetabolisme hos muse.

Som konklusion, har de klonede grise ikke en mindre variation i sammensætningen af deres tarm mikrobiota i forhold til ikke-klonede grise, i hverken den fede eller tynde tilstand. Tarm mikrobielle analyser af tynde og fede produktionssvin tyder på, at kost med højt indhold af fedt og energi, både i begrænsede mængder og *ad libitum*, er forbundet med ændringer i tarmens mikrobiota. Samlet set, påvirker kost med højt indhold af fedt og energi sammensætningen af tarmens mikrobiota anderledes end normalt føde, som det blev observeret hos både de klonede/ikke-klonede L x Y grise, Ossabaw og Göttingen minigrise.

Abbreviations

Acronym	Definition
ATP	Adult Treatment Panel
BLAST	Basic Local Alignment Search Tool
BMI	Body Mass Index
Ct	Threshold cycle value
CT-scan	Computerised Tomography scan
CVD	Cardiovascular disease
DXA	Dual Energy X-ray Absorptiometry
DIO	Diet Induced Obese
DZ	Di-Zygotic
DNA	Deoxyribonucleic acid
EGIR	European Group for the Study of Insulin Resistance
ET	Energy transfer technology
FA	Fatty Acids
FISH	Fluorescent in situ hybridization
GI tract	Gastrointestinal tract
HDL	High-density lipoprotein
HE	High-energy
HF	High-Fat
HF/HE	High-fat/ High-energy
HFD	High-fat diet
HFD-D	High-fat diet diabetic
HFD-DR	High-fat diet diabetic resistant
HOMD	Human Oral Microbiome Database
IL	Interleukin
LPS	Lipopolysaccharides
MetS	Metabolic syndrome
MZ	Mono-Zygotic
ND	NanoDrop
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PLS-R	Partial Least Square Regression
rRNA	Ribosomal Ribonucleic Acid
SCFA	Short Chain Fatty Acids

Acronym	Definition
SCNT	Somatic Cell Nuclear Transfer
SD	Standardized
T2D	Type 2 Diabetes
T2DM	Type 2 Diabetes Mellitus
TI	Terminal Ileum
TLR-4	Toll-like receptor-4
TNF-α	Tumor Necrosis Factor-alpha
T-RF	Terminal Restriction Fragments
T-RFLP	Terminal Restriction Fragment Length Polymorphism
WHO	World Health Organization

1 General introduction

Microbes are found in almost every environment and these microorganisms adapt to an ecological niche in their respective environment. The microorganisms that together make up an ecosystem are called the microbiota and the collective community of bacteria in the intestinal tract is called the gut microbiota. The gut microbiota has several functions that are important for their host, such as maintaining the mucosal integrity and providing their host with energy. However this homeostasis can be altered by diet and other external factors. Recently gut microbiota has been implicated in playing a role in development of obesity and the metabolic disorders that accompany obesity.

The work presented in this PhD thesis is based on diet-intervention study in three pig models of obesity where the relation between the composition of the intestinal microbiota and diet-induced obesity is investigated. In short, one group of pigs was cloned in order to minimize biological variations and the animals were given an experimental high-energy diet and the composition of their gut microbiota was investigated throughout the development of obesity. The two other pig models are well established models for obesity research, the Göttingen minipigs and the Ossabaw minipigs. Three papers make the basis of this thesis with the main focus of characterising the obesity related gut microbiota and these are outlined in Table 1.

1.2 Obesity

The word obese was first used in 1611 and the word obesity comes from the word *Obesitas* (Latin) meaning simply "fat". Today obesity is defined as a condition where accumulation of excess fat in adipose tissue and other organs exceeds above the normal fat deposits and can be measured by body mass index (BMI). BMI is a measurement of body fat and is calculated by dividing the weight in kilo grams (kg) with height in meters squared (m^2) ((kg)/ (height (m) ^{2})). An adult is considered overweight with a BMI greater than 25 kg/m^2 and the obese state is defined by a BMI greater than 30 (WHO, ;Flegal KM, 2010). Obesity has become a growing epidemic around the world (Figure 1) and globally obesity is inexorably rising in developing and developed countries at such a rate that the number of overweight and obese people exceeds the number of people suffering from malnutrition. In 2008 the World Health Organization (WHO) reported that more than 1.4 billion adults (Age>20) were overweight while 200 million men and 300 million women were obese worldwide and more than 40 million children under ages of 5 were estimated to suffer from overweight by 2010 (WHO). According to the health department in Denmark (Sundhedsstyrelsen) 47% of the adult population in Denmark are overweight and 13% are obese (Sundhedsstyrelsen., 2012). One of the reasons for this growing epidemic is the obesigenic environment provided by the easy access to fat-enriched, sugar-enriched, energy-dense and processed diet that are low in vitamins and micronutrient (Gross et al., 2004;Malik et al., 2010) together with a sedentary and inactive life style, whereby energy intake exceeds energy expenditure (Swinburn

et al., 1999). This state of obesity is of large economical burden due to the associated co-morbidities such as cardiovascular disease (CVD), hypertension and diabetes (Toubro S, 2 A.D.).

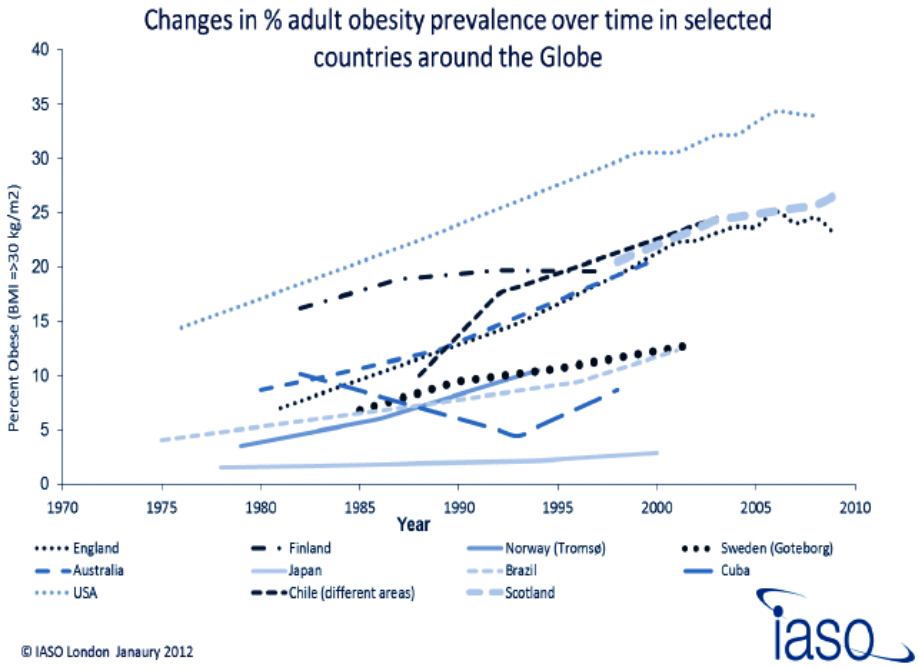

Figure 1 The Obesity prevalence in selected countries. Source: (http://www.iaso.org/resources/obesity-data-portal/resources/trends/12/)

According to WHO, obesity and overweight are the causes of 2.8 million premature deaths in adults worldwide. The dysbalance between energy intake and energy expenditure is not the only factor that results in obesity. Other factors such as a hosts' genetics, hormonal imbalances and the composition of the gut microbiota are implicated in contributing to obesity and/or promoting the state of obesity (Bäckhed et al., 2004;Bäckhed et al., 2007). Evidence supports the concept of the gut microbiota promoting weight-gain and state of obesity and this putative relation between gut microbiota and obesity has been the subject of many investigations. The new era of metagenomic techniques such as next generation sequencing has provided information about the intestinal microbiota and its' interaction with the host. This relation between obesity and gut microbiota will be described in detail in the following chapters.

1.2 Metabolic Syndrome

Obesity is accompanied by a cluster of metabolic disorders that together are called the metabolic syndrome (MetS). MetS is defined as a group of conditions that are developed along with obesity which increases the risk factor for development of type 2 diabetes (T2D), CVD and stroke (Grundy et al., 2004). The risk factors that define MetS are; central/abdominal obesity i.e. deposition of adipose tissue in viscera and around the waist and upper body, insulin resistance and ±glucose intolerance, hypertension, dyslipidemia and a systematic low grade inflammation (Grundy et al., 2004). The criteria

for MetS are slightly different according to WHO, European Group for the Study of Insulin Resistance (EGIR)(1999) and National Cholesterol Education Program Adult Treatment Panel (ATP) III (USA) (Table 2)(Bloomgarden, 2004). At least some of the risk factors must be present in a subject before the patient is diagnosed with MetS and the criteria for the presence of these risk factors vary between different organizations (Table 2).

Although obesity is a risk factor for the complications mentioned above, only some of the obese patients develop T2D, CVD or both. The subgroup of obese patients that have excess fat in viscera and abdomen are at risk of developing T2D and CVD. Some of the adverse effect of extra fat accumulation is hypertrophic intra-abdominal adipocytes causing disruption of the metabolic functions of several organs such as the liver (Musso et al., 2012). Furthermore, adipose tissue is considered to be an endocrine organ capable of producing hormones that affect metabolism and body homeostasis (Harwood, 2012). Adipocytes are also capable of producing proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin (IL)-6 (Hotamisligil et al., 1993) as well as adipocytokines such as adiponectin, leptin and resistin which play important roles in modulation of insulin (Bjorbaek and Kahn, 2004; Lehrke et al., 2004;Yang and Barouch, 2007). Impaired functions of adipocytes or their dysregulation may contribute to insulin resistance, secretion of proinflammatory adipokines, endothelial dysfunction and eventually may give rise to atherosclerosis (Teran-Garcia and Bouchard, 2007). There are several studies that suggest a genetic basis for MetS (Teran-Garcia and Bouchard, 2007;Phillips et al., 2012), however other factors such as the composition of the gut microbiota has also been implicated in playing a role in development of MetS (Zupancic et al., 2012). This putative relation between the gut microbiota and MetS will be discussed in later chapters.

Table 2 Definitions of metabolic syndrome according to WHO, EGIR and ATP III.

Criteria for MetS	WHO	EGIR	ATP III
Required Conditions			
Insulin abnormalities	Insulin resistance Impaired glucose intolerance Type 2 diabetes	Fasting hyper insulinemia	
	At least two of the following conditions (WHO, EGIR) **At least three of the following conditions (ATP III)**		
Fasting plasma Glucose		≥6.1 mmol/l	
Glucose levels			Serum glucose ≥110 mg/dl
Central Obesity	Waist-to-Hip ratio of > 0.90 (men); > 0.85 (women); BMI > 30 kg/m^2	Waist circumference >94 cm (men) and >80 cm (women)	Waist circumference >102 cm in men and >88 cm in women
Dyslipidemia	Serum triglycerides ≥150 mg/dl or HDL cholesterol < 35 mg/dl (men); < 39 mg/dl (women)	Triglycerides2.0 mmol/l or HDL cholesterol <1.0 mmol/l	Serum triglycerides >150 mg/dl HDL cholesterol <40 mg/dl (men); <50 mg/dl (women)
Hypertension	Blood pressure ≥140/190 mmHg	Blood pressure ≥ 140/190 mmHg	Blood pressure ≥130/85 mmHg

1.3 The Gastrointestinal Microbiota

The gastro-intestinal tract (GI) provides an environment that is rich in nutrients for microorganisms. The microorganisms have different functions that are necessary for the mammal host such as shaping the immunological environment, production of vitamins and digestion of complex carbohydrates (Hooper et al., 2002;Cummings, 1984). Microbiota is a term used for the microorganisms that occupy an organ and the gut microbiota is mainly referred to the microorganisms occupying the terminal ileum and large bawl (i.e. colon and cecum). The microbiota has coevolved with their hosts (Bäckhed et al., 2005) and the colonization of the mammalian GI tract begins at birth. When the animals are born naturally the first bacteria that they encounter are the maternal vaginal and intestinal microbiota and already one week after birth the number of bacteria per gram stool reaches 10^9 bacteria/ml (Palmer et al., 2007). This colonization continues and the microbial community changes over the course of time in response to diet, diseases and antibiotic treatment (Turnbaugh et al., 2009b;Dethlefsen et al., 2008). Eventually the gastrointestinal tract is colonized and ileum is reported to consist of 10^8 bacterial cells/ml and colon 10^{12} bacterial cells/ml (Figure 2) (reviewed in (Walter and Ley, 2011)). These residents of the GI tract and their collective genomes are called the microbiome (Eckburg et al., 2005;Gill et al., 2006). The normal gut microbiota is involved in many functions in the GI tract, such as preventing colonization of intestinal lumen by pathogenic bacteria, maturation of the intestine, metabolic functions, breaking down indigestible food particles, synthesis of micronutrient and immunological modulations. The products from the microbial gut community is suggested to affect obesity and obesity related metabolic disorders by influencing the intestinal function as well as other organs such as liver, brain, muscles and adipose tissue (reviewed in (Tremaroli and Bäckhed, 2012)). In this chapter the microbiota in the intestinal tract and the factors that may shape the microbial community in the intestinal tract will be presented briefly.

1.3.1 The upper digestive tract: oral, oesophagus and stomach microbiota

The upper digestive tract starts in the oral cavity where the microbiota is mainly composed of un-cultivable bacteria. There is a lower abundance and diversity at the upper digestive tract while colon consist of a more diverse and higher richness of bacteria (Sizova et al., 2012). The sequencing era has made it possible to characterize oral microbial community. According to the human oral microbiome database (HOMD.www.homd.org), the oral cavity is composed of 13 phyla with 619 different taxa. The most abundant phyla in human oral cavity are *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, TM7 and *Spirochaetes* comprising 96% of all the phyla (Dewhirst et al., 2010).
The next part of the digestive tract is the oesophagus which is a transition site and based on 16S rRNA gene analysis the oesophageal microbiota is mainly composed of six phyla, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *TM7* (Pei et al., 2004) (Figure 2). Following the oesophagus is the stomach that previously due to the low pH (1.5-2.5) environment was considered to

be sterile assuming that this acidic environment is capable of killing most of the bacteria that are ingested, however the stomach is not sterile and recently Bike et al presented the five major phyla in the stomach microbiota; *Proteobacteria*, *Firmicutes, Bacteroidetes*, *Actinobacteria*, and *Fusobacteria* (Bik et al., 2006) (Figure 2).

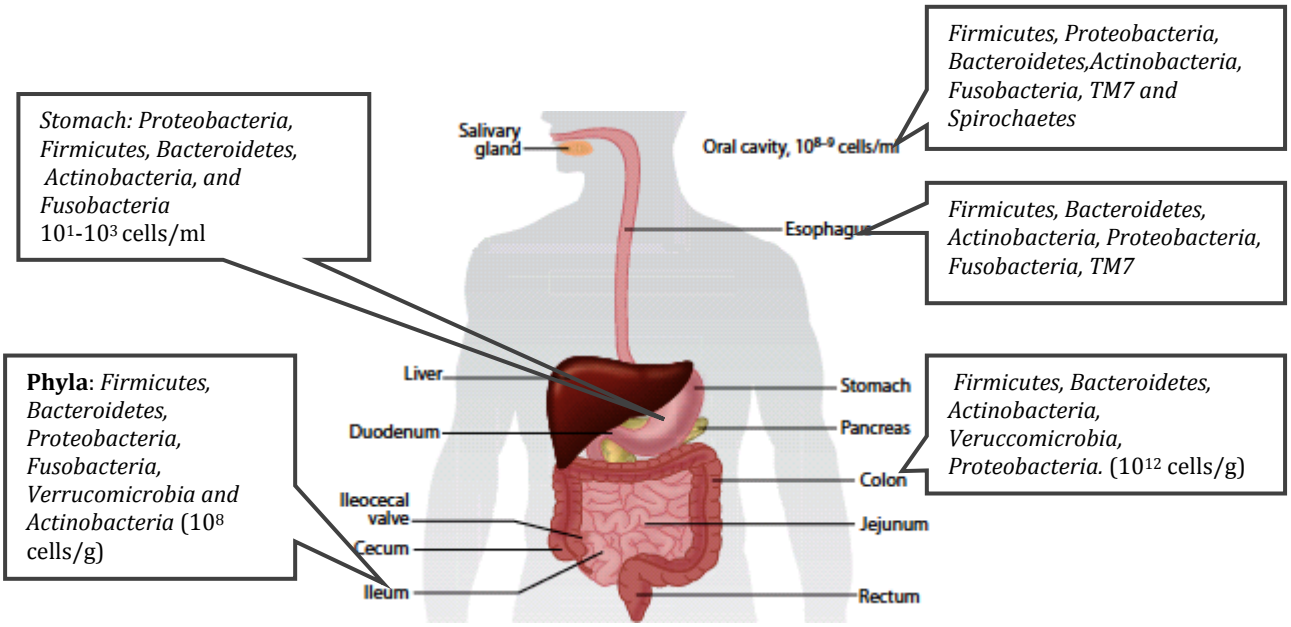

Figure 2 The microbiota of human digestive tract, the figure is adapted from Walter and Ley (2011) and modified (Bik et al., 2010;Costello et al., 2009;Pei et al., 2004;Walter and Ley, 2011).

1.3.2 The microbiota in the small intestine

The small intestine in humans consists of duodenum, jejunum and ileum which are sites of digestion, absorption and immune function. Duodenum is responsible for digestion and absorption of nutrients such as carbohydrates, polypeptides and fat by the enzyme activities while jejunum and ileum are mainly involved in absorption of nutrients and water (Thomson et al., 2003). The proximal small intestine that constitutes the duodenum (25 cm) has lower bacterial diversity than the other part of the small intestine. Here the combination of low pH that comes from the acid chyme of the stomach and the concentration of pancreatic juice, released from pancreas and bile from lever, cause an environment with low abundance of bacteria compared to the large intestine (Guarner and Malagelada, 2003). The bacterial density in the small intestine is further controlled by immunoglobulins (Ig), especially IgA which binds to epitopes of bacteria and the presence of IgA in the intestine is mainly to prevent penetration of bacteria into the mucosa (Hapfelmeier et al., 2010;Stoel et al., 2005). The small intestine is predicted to contain 10^4–10^5 bacterial cells/g in duodenum, 10^6–10^7 cells/g in jejunum and 10^7–10^8 cells/g in ileum (Booijink et al., 2007;Booijink et al., 2010). Despite efforts to investigate the microbiota of the small intestine in humans, few reports are available on the subject due to technical difficulties in sample collection from this site. Booijink *et al.* (2010) reported that the ileal effluent from humans had a

higher abundance of bacteria belonging to the orders *Lactobacillales, Clostridiales* and the Veillonella group (Booijink et al., 2010). Wang et al. (2005) reported that the jejunum microbiota was mainly dominated by *Streptococcus* and distal ileum consisted mainly of *Clostridium cluster XIVa* and *IV* and bacteria belonging to phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, *Verrucomicrobia* and *Actinobacteria* (Figure 2) (Wang et al., 2005a).

1.3.3 The large intestine and faecal microbiota

The large intestine is composed of different sections, beginning with cecum and ending in rectum. In between it is composed of the ascending colon, transverse colon, descending colon and sigmoid colon (Figure 2). Conditions in this part of the GI tract are anaerobic and many of the bacterial species are obligate anaerobes (Flint et al., 2007). The low acidic environment, longer transit time and low bile concentrations provides a very favourable environment for bacterial growth. Even though the different parts of colon are physiologically different, the composition of the microbiota of these different part at phyla level are almost the same in normal conditions (Costello et al., 2009). The colon in humans is where the fermentation of nutrients occurs by the microbiota. However, sampling from colonic microbiota is not possible without invasive approaches (Serino et al., 2012a) and therefore most of the studies on gut microbiota of humans have been performed on faecal samples.

Faecal samples are easy to obtain and many studies on human gut microbiota are based on investigations performed on these samples (Ley et al., 2006a). The faecal microbiota has been shown to be different from the colonic mucosal-associated microbiota, consisting of luminal non-adherent bacteria and some mucosal bacteria (Eckburg et al., 2005). The most abundant mucosal-associated and faecal microbiota in phyla level are *Firmicutes*, *Bacteroidetes* and *Proteobacteria* (Hong et al., 2011). Studies in humans and pigs have shown that the human and porcine colon is colonized by many different bacteria and most of the bacteria belong to the phyla *Firmicutes* and *Bacteroidetes* (Lamendella et al., 2011;Eckburg et al., 2005). The human colon, consists of 10^{11}-10^{12} bacteria/g and the most dominant phyla are; *Firmicutes*, *Bacteroidetes* and *Actinobacteria* (Eckburg et al., 2005;O'Hara and Shanahan, 2006).

1.3.4 Intestinal microbiota in pigs

There are few studies that have characterised pig gut microbiota (Leser et al., 2002;Lamendella et al., 2011). A metagenomic analysis of Yorkshire pigs' faecal microbiota revealed that *Firmicutes* and *Bacteroidetes* were the two most abundant bacteria at phyla level followed by *Proteobacteria*, *Actinobacteria* and *Spirochetes* (Lamendella et al., 2011). *Clostridiales*, *Bacteroidales*, *Spirochaetales*, *unknown gamma-Proteobacteria*, and *Lactobacillales* were the most abundant bacteria at order level. While at genus level, *Prevotella* was the most abundant genus followed by *Sporobacter* (Lamendella et al., 2011). Lamendella *et al* (2011) reported that human adult microbiota had higher ratios of *Actinobacteria* than pigs' faecal microbiota and 40% similarity at genus level to pigs' faecal microbiota

(Lamendella et al., 2011). The same group however showed that swine faecal microbiota was more similar to cow rumen and chicken than human faecal microbiota. The pig faecal microbiota revealed similarities that were closest to cow rumen in regard to *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Actinobacteria.*

In this thesis the composition of the faecal-, colonic-, ileal- (terminal ileum) and cecal microbiota are investigated in three pig models of obesity.

1.4 The Gut Microbiota of Different Phenotypes

The gut microbiota is influenced by many factors such as the hosts' genetic, diet, infections, disease, drugs such as antibiotics. Through metagenomics the microbial communities can be studied. Metagenomics is a sequence based functional analysis of bacterial community and their genomes and is used to understand the interactions of bacteria in their respective environment. This is especially interesting when studying gut microbiota in relation to certain diseases or conditions, to obtain a full understanding of the community. Sequencing has made it possible to obtain an understanding of the bacterial diversity in GI tract. Diversity is defined as the number and abundance of different types of organisms within a microbial community and diversity of the microbial community inside the gut is called the gut microbial diversity (The Human Microbiome Project Consortium, 2012). The changes in gut microbial diversity have previously been linked to conditions such as obesity (Turnbaugh et al., 2009a) and the stool bacterial community has been shown to be very diverse in healthy humans (Costello et al., 2009;The Human Microbiome Project Consortium, 2012), however the bacterial diversity may change in response to disease and dietary changes (Turnbaugh et al., 2009b).

Although bacteria are the vast majority of the microbial community with 150-200 known species (Qin et al., 2010), other organisms such as viruses and Archaea are also present in the gut microbiota (Eckburg et al., 2005;Reyes et al., 2010). Viruses such as phages, which have bacteria as their hosts (bacteriophages), have been found in faeces of humans (Reyes et al., 2010) but much less is known about the role of these viruses in the human gut and the functions they may play in different disease states such as obesity. One of the first reports on virus-induced obesity was by Lyons *et al*. in 1982 where they found a significant increase in body weight and body fat in mice infected with canine distemper virus. The virus is implicated to cause damages in hypothalamus region of the brain and cause obesity (Lyons et al., 1982), later on a relation between viral infection and obesity was reported by other groups (Bernard et al., 1999;Verlaeten et al., 2007). A correlation has been reported between adenovirus 36 and obesity in humans (Atkinson et al., 2004) and recently this correlation was found in obese children as well (Almgren et al., 2012). However, it is mainly the bacterial community in the gut that have been implicated to be connected to obesity.

1.4.1 The gut microbiota of lean phenotype

Once the humans are born, the infant gut microbiota starts to shape and is at first affected by birth method. The infant microbiota than fluctuates throughout the first year of its life and finally the microbiota converges towards the adult microbiota (Penders et al., 2006). In 2004 Gordon J. and colleagues published their findings regarding the relationship between the gut microbiota and obesity (Bäckhed et al., 2004) and by now many papers are published on the subject. The normal gut microbiota in humans consists mainly of the Gram-negative *Bacteroidetes* and Gram-positive *Firmicutes* which together constitute 90% of the total microbiota (Eckburg et al., 2005;Ley et al., 2006b). Furthermore the normal gut microbiota is characterized by high bacterial diversity (Turnbaugh et al., 2009a;Ferrer et al., 2012). Bacteria belonging to the families *Ruminococcaceae*, *Lachnospiraceae*, *Christensenelaceae* and *Peptococcaceae* were the most predominant groups found in faecal samples of lean individual (Ferrer et al., 2012). Some species of bacteria seem to be present in both lean and obese individuals namely, *Clostridium leptum*, *Clostridium coccoides* and *Bacteroides spp* (Schwiertz et al., 2009). Higher abundance of *Bifidobacterium* and *Methanobrevibacter* has been associated with lean state in humans (Schwiertz et al., 2009). In a more recent human study *Lactobacillus paracasei* and *Lactobacillus plantarum*, were found only in lean subjects while *Bifidobacteria animalis* was associated with lean state (Million et al., 2012). One study (Ley et al., 2006b) in humans on a weight loss regiment have reported a significant reduction in the abundance of bacteria belonging to the phylum *Firmicutes* and an increase in abundance of bacteria in the phylum *Bacteroidete*. However a more recent study contradicts these findings where the subject with normal weight had lower abundance of *Bacteroidetes* (22.9%) than the overweight (46.8%) and obese subjects (45.0%) (Schwiertz et al., 2009). Surgical interventions such as gastric bypass have been an effective way of obtaining the lean state in humans resulting in substantial weight-loss in morbidly obese humans (Zhang et al., 2009). Gut microbiota of post gastric bypass patients have shown distinct differences between lean and obese individuals with lower abundances of *Firmicutes* and proportional increase in Gamma *Proteobacteria* (Zhang et al., 2009).

There are several explanations for the conflicting reports on bacterial composition in the gut microbiota in lean state. Some of the plausible reasons could be differences in methodological approaches to characterize the gut microbiota, furthermore factors such as diet, genetic background and age may have contributed to the differences observed in different studies.

1.4.2 The gut microbiota of obese phenotype

The role of gut microbiota in development of obesity has been investigated for a long time and correlation with obesity and alterations in the composition of the gut microbiota has been reported (Bäckhed et al., 2004). One of the early studies on the relation between obesity and gut microbiota was in an obese mouse model, C57BL/6, with homozygous mutation of the leptin gene (ob/ob), reared

germ-free and subsequently conventionalized i.e. the intestine of the germ-free mice were inoculated with gut microbiota of conventional mice (Bäckhed et al., 2004). Conventionalisation caused weight-gain and increase in fat-mass in the mice despite decreased food intake and the gut microbiota was implicated as an environmental factor playing a role in obesity (Bäckhed et al., 2004). Studies in mice, pigs and humans have reported that the body-fat composition correlated with the abundance of the two largest phyla in the gut, *Firmicutes* and *Bcteroidetes* (Ley et al., 2005;Ley et al., 2006b;Turnbaugh, 2006;Guo et al., 2008a). However this relation between obesity and abundance of *Bacteroidetes* and *Firmicutes* is controversial and therefore it is important to characterize microbial community on lower taxonomic levels.

Higher ratio of *Firmicutes* to *Bacteroidetes* has been reported in a state of obesity (Ley et al., 2006b) while other studies have reported the opposite (Schwiertz et al., 2009;Zhang et al., 2009). A recent study has shown clear differences between lean and obese gut microbial community. In the obese individuals' gut microbiota, a lower bacterial diversity was observed and the gut microbiota was dominated by phyla *Firmicutes*, class *Clostridia*, family *Lachnospiraceae* and *Ruminococcaceae* (Ferrer et al., 2012). Obesity is a multi-factorial condition and the connection between obesity and gut microbial community is still under investigation. Diet has been implicated to have a great effect in shaping the gut microbial community (Hildebrandt et al., 2009;Semova et al., 2012). Several studies have revealed that high-fat/high-energy (HF/HE) diet changes the gut microbiota with the bloom of some bacteria in expense of other "beneficial" bacteria, thereby turning the gut microbiota into an energy harvesting environment (Hildebrandt et al., 2009;Jumpertz et al., 2011).

The complex carbohydrates that are not digested in the small intestine are converted to short chain fatty acids (SCFA) in colon by the fermentation action of the bacteria and the SCFA are then taken up by the host (Blaut and Clavel, 2007). Metagenomic studies have revealed that the expression of certain genes in the bacteria can contribute to extraction of energy in form of SCFA from digestion of otherwise indigestible carbohydrates. The SCFAs are then absorbed by the intestine and are converted to lipids in lever that are further accumulated in adipocytes (Bäckhed et al., 2004).

Dietary fats such as triglycerides are digested in the intestinal lumen by the action of lipases and free fatty acids (FFA) are released. In the intestinal epithelium, these FFA are absorbed by eneterocytes. Three fates await the FAs, i) FAs are oxidized (energy is generated), ii) FAs are released in circulation as FFA or in form of chylomicrones, iii) FAs are re-esterified into triglycerides (Iqbal and Hussain, 2009). The FFAs in circulation are eventually transported to other organs for either storage or oxidation. Recently a connection between increased FA absorption and gut microbiota upon feeding was observed in Zebrafish(Semova et al., 2012). Especially bacteria belonging to phylum *Firmicutes* was bloomed upon feeding (Semova et al., 2012). Hildebrandt *et al*. found distinct community changes in the gut

microbiota of RELMβ knockout mice (RELMβ KO) upon switch from standard chow to HF/HE chow (Hildebrandt et al., 2009). Expression of the RELMβ gene is specific in colonic goblet cells and are expressed when the intestinal tract is colonized by bacteria (Wang et al., 2005b). Alteration in diet normally affects expression of RELMβ but the RELMβ knockout mice that received high-fat diet (HFD) remained relatively lean as compared to wild type mice (Hildebrandt et al., 2009). The HFD caused changes in the gut microbiota of these mice independent of the state of obesity (Hildebrandt et al., 2009). Furthermore they found a bloom in *Clostridia* and *Proteobacteria*, class *Delta Proteobacteria*, more specifically bacteria belonging to the genus *Desulfovibrio* (Hildebrandt et al., 2009). Altered nutrient load have shown to induce changes in the gut microbiota of humans as well. A 20% increase in *Firmicutes* was associated with increase in nutrient absorption corresponding to ≈150 kcal while a 20% increase in *Bacteroidetes* was associated with a decrease in nutrient absorption and stool energy loss (Jumpertz et al., 2011).

Treatment of mice with antibiotics have been shown to reduce the abundance of both *Firmicutes* and *Bacteroidetes* and increase the abundance of *Proteobacteria* (Murphy et al., 2012). Interestingly, in this study the HF fed mice gained less weight upon ingestion of antibiotics, (Vancomycin) indicating that the gut microbiota can be manipulated therapeutically and such findings may be used to manage obesity (Murphy et al., 2012). The connection between diet, obesity and gut microbial community has long been investigated and the metagenomics have clarified some of the paradigms connected to this subject. Another function of the gut bacteria is to utilize substrates such as mucin when the gut environment is lacking the fermentable carbohydrates due to the ingestion of diets devoid of fibre. This utilization causes the bloom of some bacteria at the cost of other bacteria. The effect of Western diet on the gut microbiome was investigated using metagenomic analysis of the cecal microbiota in obese mice which revealed an increase in the genes encoding enzymes utilized in digestion of indigestible polysaccharides (Turnbaugh et al., 2008). Furthermore the Western diet was associated with bloom of a group of bacteria, the *Mollecutes*, at the expense of *Bacteroidetes* in cecal microbiota which also contains genes involved in cell wall biosynthesis (Turnbaugh et al., 2008). Together these studies have shed light on the hidden world of gut microbiota, however much is to be learned about the gut microbiota and its interaction with their host.

1.4.2.1 The gut microbiota of obese pigs

The gut microbiota in obesity has also been studied in obese Landrace and Meishan pigs and it was shown that the proportions of *Bacteroidetes* negatively correlated with body fat (Guo et al., 2008b). Lower abundance of *Bacteroidetes* was observed in obese pigs as compared to lean pigs; however obese Meichan pigs had significantly lower proportions of *Bacteroidetes* than Landrace pigs. Furthermore, it was shown that body fat did not correlate with the abundance of *Firmicutes*, which have otherwise

shown to be increased in other obesity experiment in pigs (Guo et al., 2008a). It must be mentioned that in different studies the diet is not the same, which could explain the conflicting results in these studies.

1.4.3 The gut microbiota and metabolic syndrome

Diet-induced obesity is connected to metabolic impairments such as T2D and CVD. Obesity related T2D, "diabesity", is characterized by low grade inflammation. It is mainly this low grade inflammation that has been implicated in the development of T2D and CVD (Creely et al., 2007;Després, 2012). Some of the early studies on the link between gut microbiota and inflammation revealed a correlation between the gut microbial community and insulin resistance (Hotamisligil, 2006). Visceral adiposity is implicated to be related to metabolic dysfunction and low grade chronic inflammation which contributes to insulin resistance and CVD (Lee et al., 2009a). Studies in diet induced obese mice have shown a constant but low increase in plasma lipopolysaccharides (LPS) in response to HF diet (Cani et al., 2007b). LPS are large glycolipids and are located at the outer membrane of Gram-negative bacteria. LPSs are also called endotoxins and are released when the bacteria are disintegrated. LPS binds to Toll-like receptor-4 (TLR-4) which is a transmembrane glycoprotein that recognises LPS's lipid anchor and initiates secretion of proinflammatory cytokines.

Cani and colleagues (Cani et al., 2007b) observed a dramatic increase in the plasma concentrations of LPS in mice that were fed a HF diet for a duration of four weeks and found that *Bacteroides* were significantly reduced and Gram-positive bacteria were increased in the gut microbiota of these mice. Subsequently, they fed a group of mice orally with LPS and water and another group of mice with LPS and oil to see if it was the HF diet that caused this increase in plasma LPS. They found that mice fed oil with LPS, had higher levels of LPS in their plasma, suggesting dietary fat as a factor that could play a role in an increased in plasma LPS. In this study, the blood plasma levels of LPS was low but constant, unlike in sepsis, and this phenomenon was called metabolic endotoxemia (Cani et al., 2007b).

The absorption and transport of LPS from the intestinal lumen is thought to be facilitated by high fat concentrations in the gut and a mechanism through chylomicrones (Ghoshal et al., 2009). A more recent study in mice suggests that HF diet causes metabolic changes that affects gut permeability in proximal colon (Lam et al., 2012). In brief; the hypothesis is that in the circulation, LPS binds to TLR-4 that reside on the surface of immune cells and induce an inflammatory cascade that eventually results in insulin resistance and other metabolic dysfunctions (reviewed in (Cani and Delzenne, 2011)). The HF feeding in these studies caused a reduction in the abundance of *Bifidobacteria*. These bacteria are reported to reduce endotoxins in the intestine and have shown to improve HF-diet induced obesity and diabetes (Figure 3)(Cani et al., 2007a;Griffiths et al., 2004). It was suggested that this increase in LPS may cause increase in lipogenesis, stenosis and decreased insulin sensitivity, in adipose tissue it may cause increase in lipopolylipase activity, macrophage infiltration and increased inflammation, in muscles it may be the cause of decrease in insulin sensitivity (Figure 3)(Cani et al., 2008). These studies together

suggest that gut microbiota plays an important role in development of metabolic disorders as observed in diet-induced obese subjects.

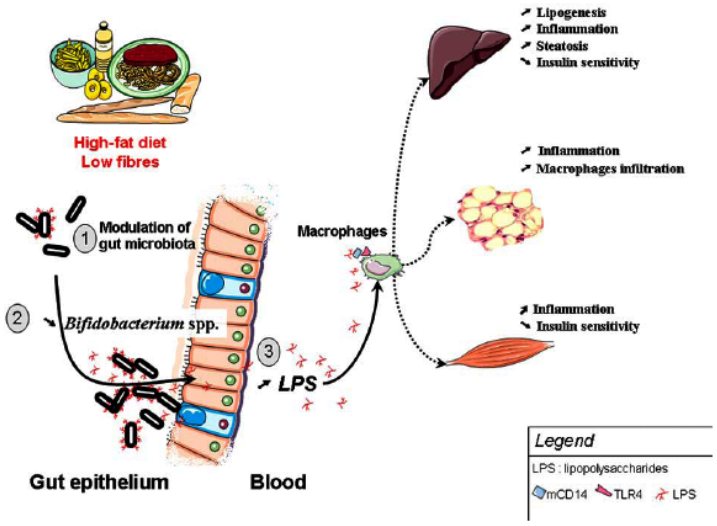

Figure 3 Pathways that may cause metabolic endotoxemia through high-fat diet feeding. HFD is proposed to modulate the gut microbiota and change the intestinal barrier by causing a leakage of LPS which through CD14/TLR4 dependent pathway affects the lever, adipose tissue and muscles. The figure is adapted from (Patrice D.Cani. and Nathalie M.Delzenne., 2009).

To observe the changes in the gut microbiota in T2D, Serino *et al*. (2012) used mice that were fed a HFD. In this study diabetes was induced in one group of mice (HFD-D) while the other group was diabetes resistant (HFD-DR) (Serino et al., 2012b). The gut microbial profile of the diabetic mice was associated with increased endotoxemia due to increased gut permeability (Serino et al., 2012b). These changes in the gut microbiota was characterized by a significant reduction in genus *Oscillobacter* and family of *Lachnospiraceae* (*Firmicutes*) in HFD-D mice (Serino et al., 2012b). Furthermore *S24-7* family belonging to phyla *Bacteroidetes* was tripled in diabetic mice (Serino et al., 2012b). Interestingly, another study in mice fed a HFD diet also reported a dramatic and significant increase in the abundance of bacteria belonging the *Oscillobacter* genus as compared to mice fed a control diet (Lam et al., 2012). In both studies the gut permeability was increased while Lam *et al*. showed an increase in the expression of TNF-α in the colon tissue resulting in systematic insulin resistance (Lam et al., 2012).

There are few studies on the relation between the gut microbiota and T2D in humans and these reports are conflicting. Larsen *et al*. have recently reported that in humans with T2D the gut microbiota was altered and characterized by a lower abundance of *Firmicutes* and higher abundances of *Bacteroidetes* and *Proteobacteria* as compared to non-diabetic controls (Larsen et al., 2010). They found a positive correlation between an increase in bacteria belonging to *Prevotella* and blood glucose levels while the proportions of *Clostridia* was significantly lower in diabetic humans (Larsen et al., 2010). Another study in T2D and non-diabetic humans reported an association between *Bacteroides* in the gut microbiota of diabetic subjects and *Prevotella* was the most abundant genus in the non-diabetic subject (Wu et al., 2010) which is opposite of the findings by others (Larsen et al., 2010). Many factors could explain these

conflicting reports, such as, methodology, patient background, genetic background and diet and therefore the relationship between T2D and gut microbiota needs to be investigated further in larger cohorts.

2 Animal models in obesity studies

Animals are generally preferable models for multi-disciplinary studies aiming to understand human diseases. It is more practical to use animal models in understanding the underlying causes of a condition or disease as it is possible to perform more invasive experiments on animals as opposed to humans. Another limitation in human studies is the ethical issues. Even though animal experiments may provide relevant knowledge about the response of a treatment or cause of a disease; still it should be kept in mind that there is no direct equivalence between humans and animals.

Rodents are the experimental animal models that are used more frequently due to their size and the fact the they are less costly than larger animals, however there are many differences between rodents and humans (Litten-Brown et al., 2010). Most of the rodent models used in relation to gut microbiota and obesity are transgenic mice such as *ob/ob* mice with homologous mutation in leptin receptors, making it possible to induce the obese phenotype in these mice (Zhang et al., 1994). Diet Induced obese rodent models (DIO mice) of C57-black-6 strain (C57BL/6J) are also frequently used in obesity studies. DOI mice are able to gain substantial amount of weight when fed HFD with visceral adiposity, insulin resistance and hyperinsulinemia (Petro et al., 2004). Other rodent models of obesity are mice that have mutation in a gene involved in toll-like receptor (TLR)-mediated bacteria–host interactions, the MyD88 knockout mice (MyD88-/-)(Kawai et al., 1999). Although there are several knockout mice that are able to exhibit obesity, spontaneous development of atherosclerosis and insulin resistance do not occur in rodent models as they do in humans, mainly due to metabolic differences between humans and rodents (Brindley and Russell, 2002) (reviewed in (Varga et al., 2010)). Research in metabolic disorders related to obesity therefore require animal models possessing similar metabolic and physiological phenotypes as humans in order to be able to translate the findings from animal models to humans.

The experiments regarding nutrition and GI tract have been mainly performed in nine species of mammals other than humans (mice, rat, guinea-pigs rabbit, cat, dog, sheep, pig and cow) (Guilloteau et al., 2010). Even though GI tract and diet of mice is very different from that of humans, mice are usually the animals chosen for experimental studies due to their availability, low cost and low housing cost as compared to larger animals such as pigs. Pigs may provide better animal models than mice in GI research as their GI tract t possess more similarities to humans than the rodent models (Kararli, 1995). Furthermore, pigs are often used in biomedical research as their GI anatomy (Figure 4), metabolism and physiology are very similar to that of humans (Guilloteau et al., 2010).

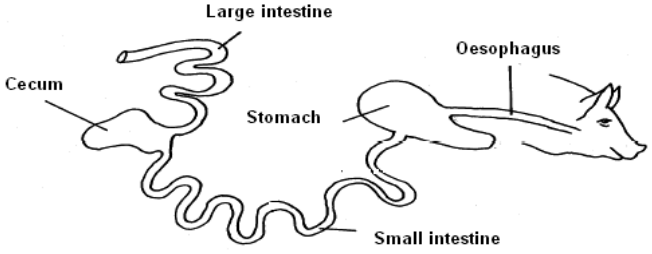

Figure 4 The pig gastrointestinal tract. (www.thepigsite.com)

Additionally due to the fact that pigs are omnivores, their diet requirement resembles that of humans making them suitable for diet-intervention and obesity related studies (Miller and Ullrey, 1987). Pigs may therefore provide a preferable animal models due to their large size, their ability to adapt to humanized nutrition and large GI anatomy, making it easier to perform biomedical and surgical intervention studies such as diet intervention and gastric bypass surgery (Lee et al., 2003;Escareno et al., 2011).

The pigs that are mainly studied in connection with MetS posses either lean or obese genotype. The lean genotypes are mainly domestic pigs that are developed by selective breeding through many generations in order to display lean meat for the meet industry. This type of pig may become obese when fed high-energy diet *ad libitum* and may express atherosclerosis. However insulin resistance and T2D are not developed naturally in these pigs but can be induced through administration of a β-cell destructive drug (Spurlock and Gabler, 2008;Koopmans et al., 2011).

The obese genotype of pigs are different breed of pigs that are selected for their potential in obesity and MetS research such as, Mexican hairless pigs, Göttingen minipigs and Ossabaw minipigs in which atherosclerosis can be induced through diet (Camacho-Rea et al., 2010;Bellinger et al., 2006). Recently Mexican hairless pigs have been evaluated for their potential in obesity and diabetes research (Camacho-Rea et al., 2010), while Ossabaw and Göttingen minipigs have been studied extensively in research related to obesity, CVD and T2D (Bellinger et al., 2006;Litten-Brown et al., 2010).

This Ph.D. thesis is based on experiments performed on three different breed of pigs, used as models for diet-induced obesity and gut-microbiota, one of them being a well-established model of obesity, the "Göttingen minipigs"(Val-Laillet et al., 2010a) and one a model for MetS "Ossabaw minipigs" (Litten-Brown et al., 2010;Val-Laillet et al., 2010b). These three pig models will be described briefly in this chapter.

2.1 Landrace x Yorkshire Pigs

The Yorkshire-crossed strains are known for their large size and lean frame but CVD and T2D are not induced naturally through diet and obesity (Bellinger et al., 2006). The domestic Landrace x Yorkshire (L x Y) pigs are generally used as production pigs in Denmark as well as many other countries, however they are also used as experimental animal models (Jonsson et al., 2006;Camacho-Rea et al., 2010). The L x Y cross pigs are normally of lean phenotype but obesity can be induced through diet-intervention. It is challenging to induce insulin resistance or metabolic syndrome in L x Y pigs through diet-induced obesity (Camacho-Rea et al., 2010). In a recent study, Mexican hairless pigs and L x Y pigs were evaluated for their potential as models for obesity-related metabolic disorders (Camacho-Rea et al., 2010). The L x Y were not considered a good model of obesity, as there were no changes in the glucose tolerance or amount of fat deposits by *ad libitum* feeding as compared to the Mexican hairless pigs. The contemporary domestic pigs such as L x Y pigs do not develop metabolic disorders related to obesity

but when fed substantial amounts of high-fat diet and cholesterol they have the potential of becoming humanoid models for atherosclerosis (Koopmans et al., 2011). Taken together, L x Y pigs may provide a model of obesity but not for obesity related metabolic disorders (Table 3).

2.2 Cloned Pig Model

The L x Y pigs for this study were cloned by somatic cell nuclear transfer (SCNT) (Figure 5). In this method the nucleus of somatic cell from a donor animal is transferred into an enucleated oocyte obtained from a donor animal and the cell is subsequently implanted to the surrogate mother (Figure 5). Cloning of pigs by SCNT is a common cloning method (Archer et al., 2003;John et al., 2005;Park et al., 2005;Hwang et al., 2009) but the method is not efficient and results in low numbers of cloned animals due to loss of embryos during the gestation period or the death of newborn piglets shortly after birth (Park et al., 2005;Whitworth et al., 2009).

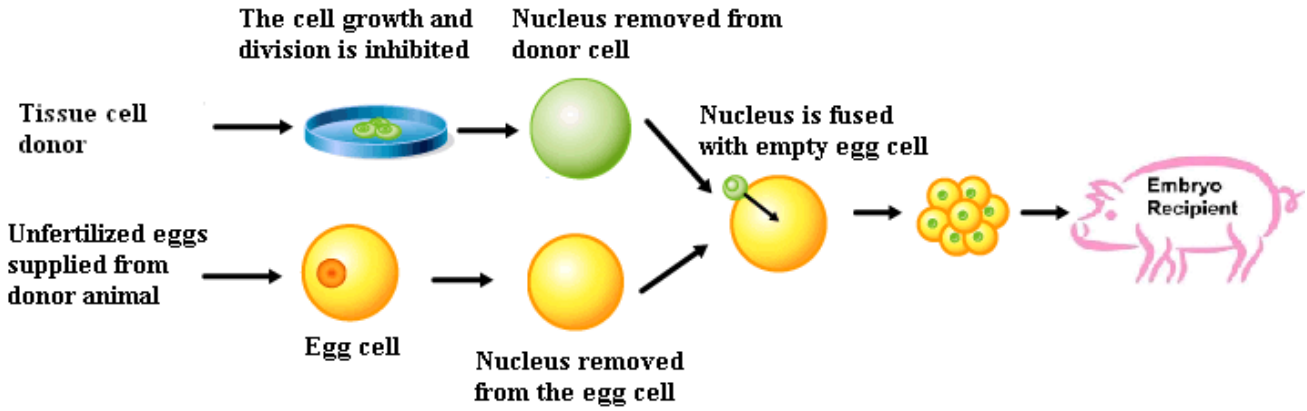

Figure 5 Cloning by somatic cell nuclear transfer. The somatic cells are harvested from tissue cell donor and the cells are inhibited to divide and grow. Unfertilized eggs are obtained from donor animal and the cell nucleus is removed. This enucleated oocyte will receive the nucleus from somatic cell and the cell is implanted to surrogate mother 5-7 days after cloning.

Cloning may affect the phenotype of the cloned animals giving rise to altered metabolism (Clausen et al., 2011;Christensen et al., 2012), skin abnormalities (Figure 8) (Hwang et al., 2009)and other physiological defects (Tian et al., 2008). Despite all these drawbacks, cloning may provide a favourable animal model firstly by being genetically identical and secondly if biological variations are reduced, in which case low number of animals can be used in biological experiments. However it was found, that the biological and inter-individual variations are not reduced in cloned animals (Clausen et al., 2011;Rødgaard T et al., 2012), this is discussed in **Paper I** and **II**.

2.3 Göttingen minipigs

In the 1960s Göttingen minipigs were developed by professor Fritz and colleagues at the University of Göttingen, Germany (Simianer and Köhn, 2010). The Minnesota Minipigs, which were already experimental animal model for pharmacological and medical research at Hormel Institute in Austin,

Minnesota (USA), were crossed with Vietnamese potbelly pigs from the Wilhelma Zoo in Stuttgart, Germany (Simianer and Köhn, 2010). This cross was then crossed again with commercial German Landrace pigs by artificial insemination which subsequently resulted in Göttingen minipigs, represented by 60% Vietnamese potbelly pigs, 33% Minnesota Minipigs and 7% German Landrace (Simianer and Köhn, 2010). Eventually Göttingen minipigs became available across Europe and are now also used in USA for experimental studies in pharmacology. They are one of the large experimental animal models used in obesity and metabolic syndrome studies (Larsen et al., 2005;Larsen et al., 2002;Christoffersen et al., 2007). Göttingen minipigs are easier to handle in the laboratory settings due to their smaller size than conventional pigs. To verify whether the Göttingen minipigs were suitable as animal model for obesity and metabolic disorders, Johansen *et al*. (Johansen et al., 2001) did a metabolic assessment of lean and obese Göttingen minipigs. There are some early studies on atherosclerosis (Jacobsson LS et al., 1994) and hypertension (Mey J et al., 1984) in Göttingen minipigs, however it was not until 2001 that Jacobsen and colleagues attempted to characterize the obesity related metabolic disorders in these animal. A marked gain in body-weight in form of fat was observed in the minipigs when fed a HFD and HE diet. This increase in adipose tissue was 52% more than the lean controls and only 28% of the gain in body-weight was lean tissue while the rest was fat (Figure 6)(Jacobsson LS et al., 1994). Although blood glucose levels were not different between lean and obese minipigs, the obese Göttingen showed evidence of insulin resistance when compared to their lean counterparts. Furthermore high blood lipid and cholesterol levels have been observed in Göttingen minipigs on a HFD (Jacobsson L, 1989). In 2007 Christofferssen *et al*. found differences in MetS parameters between male and female Göttingen minipigs (Christoffersen et al., 2007). The female Göttingen minipigs gain more weight on the same diet as compared to their male counterparts.

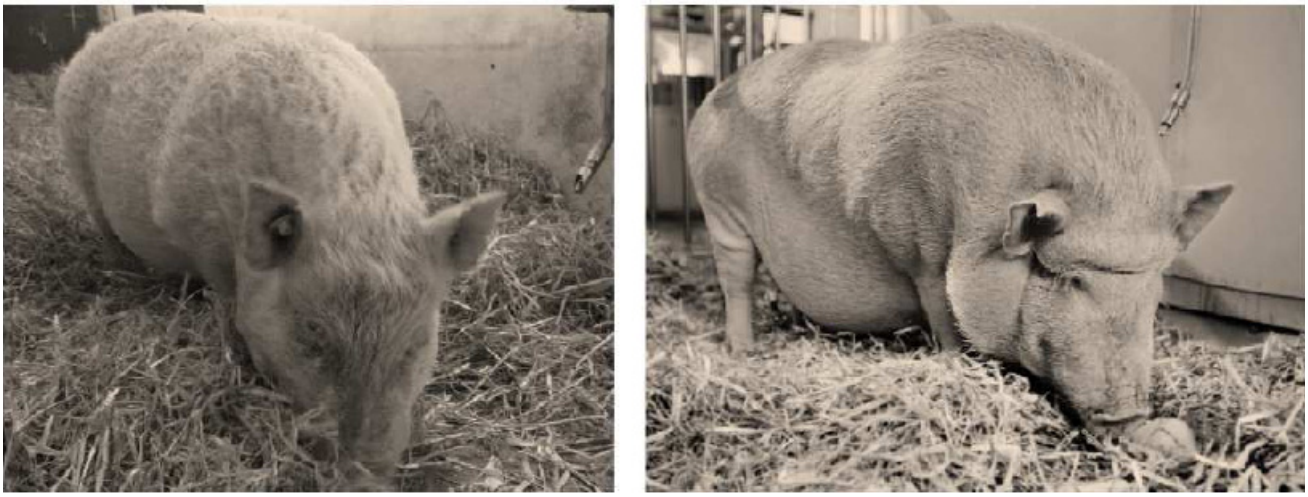

Figure 6 Göttingen minipigs of lean (left) and obese (right) phenotype. Courtesy of NOVO NORDISK A/S and University of Copenhagen.

The female Göttingen minipigs are more atherogenic and insulin resistant and therefore provide a better animal model for MetS than the male minipigs (Christoffersen et al., 2007). It is suggested that

male Göttingen minipigs are more protected against obesity and MetS than the females, mainly due to high concentrations of testosterone which protect them against metabolic diseases (Christoffersen et al., 2007;Christoffersen et al., 2010). The food intake in male Göttingen minipigs was increased corresponding to an increase in body-weight and fat upon castration. Furthermore, a significant decrease in testosterone levels indicates a relation between this hormone and regulation of food intake, body fat percent, body weight and metabolic parameters as shown in humans as well (Mystkowski and Schwartz, 2000;Wang et al., 2011). Together these observations suggest that Göttingen minipigs are suitable models for research in obesity and the MetS.

2.4 Ossabaw minipigs

In the 1500s Spanish explorers brought the now called Ossabaw miniature swine (minipigs) to the Ossabaw Island located off the cost of Georgia in USA. Since then the Ossabaw minipigs have remained in the island without being able to migrate due to the ocean, separating the island from the mainland (Sturek et al., 2007). The Ossabaw minipigs became isolated on the Ossabaw Island and had to survive at periods of famine. The pigs feasted during periods of abundance and stored large amount of fat in their body to be later used during times of famine and thereby obtaining a "thrifty genotype" (Sturek et al., 2007).

The Ossabaw minipigs were studied during 1970s and 1980s but were then forgotten and rediscovered in 2001 (Bellinger et al., 2006). In 2002 a breeding colony was established from the Ossabaw minipigs that were obtained from the Ossabaw Island (Sturek et al., 2007). These animals were subsequently studied for metabolic disorders accompanying obesity (Dyson et al., 2006;Bratz et al., 2008;Lee et al., 2009b;Bell et al., 2010). Ossabaw minipigs provide a suitable model for metabolic syndrome as they develop all the pathological aspects of MetS when fed atherogenic diet at longer durations (Table 3) (Litten-Brown et al., 2010).

Table 3 Features metabolic syndrome present in the three animal models investigated in this thesis.

Metabolic features	Domestic (L x Y)	Göttingen	Ossabaw	References
Obesity	Yes	Yes	Yes	(Sturek et al., 2007;Dyson et al., 2006;Bratz et al., 2008;Johansen et al., 2001;Christoffersen et al., 2010;Larsen et al., 2002;Val-Laillet et al., 2010b)
Insulin resistance	No	-	Yes	(Christoffersen et al., 2007;Val-Laillet et al., 2010a;Lee et al., 2009b;Lee et al., 2003)
Glucose intolerance	No	-	Yes	(Val-Laillet et al., 2010a;Lee et al., 2009b)
Dyslipidemia	No	Yes	Yes	(Johansen et al., 2001;Lee et al., 2003)
Hypertension (arterial)	No	Yes	Yes	(Mey J et al., 1984;Kreutz et al., 2011;Lee et al., 2009b)
Atherosclerosis	No	Yes	Yes	(Jacobsson L, 1989;Edwards et al., 2010;Kobari et al., 1991)
CVD	No	-	Yes	(Neeb et al., 2010;Lee et al., 2003)

Ossabaw minipigs are therefore recognized as being the large animal model that provide a good model for MetS (Dyson et al., 2006;Sturek et al., 2007). Comparisons between Ossabaw and Yucatan swine have shown many differences between the two races of pigs in development of MetS. Compared to Yucatan pigs, Ossabaw minipigs have shown to have a greater increase in at least four MetS features including obesity, glucose intolerance, hyperinsulinemia and increased arterial pressure (Neeb et al., 2010). It has been reported that Ossabaw minipigs that were fed high caloric-atherogenic diet became morbidly obese and developed elevated cholesterol, arterial hypertension and insulin resistance (Kreutz et al., 2011).

Figure 7 Ossabaw minipigs, lean and obese phenotype.

The Ossabaw minipigs' ability to become morbidly obese on HF/HE diet and progression to insulin resistance and occurrence of MetS makes them uniquely suitable for obesity-MetS studies (Bratz et al., 2008). So far there have been no studies on the MetS related gut microbiota in Ossabaw minipigs and this study is the first to characterized the gut microbiota in lean and obese Ossabaw minipigs.

3 Hypotheses

This Ph.D. thesis is based on three studies, addressing obesity issue and the relation between obesity induced by HF/HE human-like diet, genetic background and composition of the intestinal microbiota in cloned pigs and non-cloned pigs of the same genetic background. Furthermore, the relation between adiposity and metabolic syndrome was investigated in two well-established pig models of obesity namely the Göttingen minipigs, and the Ossabaw miniature pigs.

The research hypotheses for this Ph.D. thesis were:

1. Does cloning minimize the biological variation among pigs, thereby making cloned pigs a more suitable animal model for gut-microbiota studies (**Paper I**)?
    - Hypothesis 1: The cloning of pigs minimises biological variations and thereby a more uniform profile of gut microbiota would be obtained. Furthermore cloning reduces the need of large numbers of animals and gives a study the statistical power which otherwise would be obtained by a large number of animals.

2. How does the gut microbial community respond to HF/HE diet over time from a state of leanness to the obese state (**Paper I**)?
    - Hypothesis 2: Specific groups of bacteria in the gut microbiota bloom in response to either the state of obesity or HF/HE diet, or both.

    - Hypothesis 3: There is a change in the ratio of the phyla *Firmicutes* to *Bacteroidetes* in response to diet and adiposity as shown before, however this change would be gradual.

3. Is there less biological variation between the lean cloned pigs (**Paper II**)?
    - The lean cloned pigs display a different phenotype (based on their gut microbial profile), in that the state of obesity does not camouflage the identical phenotype which is expected in genetically identical animals.

4. What are the differences between the microbiota of colon and terminal ileum of lean and obese pigs and which bacteria are specific to which part of the intestinal tract in obese and lean state?
    - Hypotheses 4: The microbiota of proximal colon and terminal ileum are different at normal metabolic and physical condition such as the lean healthy state. We then hypothesized that the colonic and ileal microbiota are different between lean and obese pigs (**Paper II**).

5. Is it the diet or development of adiposity that effects the composition of the gut microbial community (**Paper II**)?
    - Hypotheses 5: We hypothesized that the gut microbiota in lean animals differs from obese animals and thereby the state of obesity affects the gut microbiota.

6. What are the differences between the gut microbiota of obese pigs on normal chow and obese pigs fed atherogenic diet and suffering from metabolic syndrome? Do pigs with metabolic syndrome display phenotypic specific intestinal microbiota (**Paper III**)?
    - Hypothesis 6: Excessive feeding and obesity causes changes in the gut microbiota that is distinct from the effect of atherogenic diet.
    - Hypothesis 7: The gut microbiota of obese pigs with metabolic syndrome displays a specific profile that is different from that of their lean counter parts without metabolic syndrome (Ossabaw minipigs).

4 General materials and methods

4.1 Animal Experiments

This section is composed of a description of the animal models and the different methods used in this study.

4.1.1 Nutriomics Project

The Danish Landrace and Yorkshire cross pigs were used in a large nutriomic study with several other groups doing obesity related research in collaboration with the University of Aarhus, Foulum (Tjele, Denmark) which was supported by a grant from the Danish Strategic Research Council. The aim of the Nutriomics project was to obtain a pig model for nutriomic study in multiple disciplinary areas of nutrition, functional food and gene interaction. Pigs were cloned in order to minimize genetic and biological variation and to determine if cloned pigs could provide a better model for nutritional studies than normally bred pigs. Another part of the Nutriomic study was to evaluate the effect of functional food such as pre- and probiotics on obesity. The aim was to produce 240 cloned pigs, of which 120 were cloned domestic pigs (lean phenotype) and 120 were cloned pigs of obese phenotype, although this came to be difficult in practice. Due to complications of the cloning process only 17 cloned domestic pigs were produced. At the beginning of the project the aim was to clone Mangalica Hungarian pigs of obese phenotype (Figure 8); however the cloning of this phenotype failed. Furthermore, the effect of functional foods such as pre- and probiotics on obesity and metabolic syndrome was to be investigated in obese cloned and non-cloned pigs due to low number of animals this part of the project was not conducted. At the beginning of the nutriomics project the aim was to clone Mangalica Hungarian pigs of obese phenotype (Figure 8). Even though Mangalica are able to obtain the obese phenotype through diet, the presence of obesity related metabolic disorders are not known; furthermore the cloning of this phenotype was failed.

Figure 8 The pig models used and were considered to be used in nutriomics project. Cloned Landrace x Yorkshire from Nutriomics project (I), The Mangalica Hungarian pig (II) and the Yucatan minipig (III)

Therefore the Yucatan mini pigs were chosen to replace Mangalica, however they are a leaner phenotype and although obesity can be induced in Yucatan minipigs by selective breeding through

generations, naturally they are unable to exhibit obesity or develop obesity related metabolic disorders such as insulin resistance and hypertension (de Smet et al., 1998;Litten-Brown et al., 2010). In nutriomics project 10 Yucatan minipigs were successfully cloned, of which five were allocated as the lean phenotype and five as obese. Only seven non-cloned Yucatan pigs were produced (3 obese and 4 lean) and therefore due to low number of animals in each group and the above mentioned reasons, we chose not to use Yucatan minipigs in our study.

4.1.2 Cloned and non-cloned Landrace x Yorkshire pigs of Obese and Lean Phenotype

The cloning experiments for the studies in this thesis were performed by somatic cell nuclear transfer as mentioned before (Figure 5) and the donor cells were obtained from a 65% Landrace x 35%Yorkshire (65% L x 35% Y) sow as described previously. Five to six days after cloning the cloned embryos were transferred surgically to surrogate sows (recipients) (Kragh et al., 2004;Clausen et al., 2011). The cloned and non-cloned L x Y pigs were produced in duration of three years. From the first cloning experiment (Exp I) four live female cloned pigs of 65% L x 35% Y were produced. Three female non-cloned pigs of 36% L x 64% Y and one female control pig of 75% L x25% Y were allocated as the control group for the cloned pigs (Figure 9). All the pigs from this experiment (Exp I) were born naturally, however three clones were lost during vaginal birth and it was decided that the following sets of cloned pigs were to be delivered by caesarean section (C-section). From the second cloning experiments (Exp II), five cloned pigs and six non-cloned pigs were born by C-section. Pigs from experiment I and II were allocated as the obese group. In the last cloning experiment (Exp III) eight female cloned pigs and nine non-cloned pigs were born naturally. Figure 9 shows a detailed experimental set up in the three experiments performed in this study and the time line of nutriomics animal experiments. All the pigs in the experiments were weaned at 28 days of age and subsequently fed a standard pig-diet (Table 4).

Table 4 Energy distribution in the experimental feed for Domestic pigs, Göttingen and Ossabaw minipigs.

	Domestic pigs		Göttingen minipigs		Ossabaw minipigs	
	Transition diet after weaning	Lean/obese	Lean	Obese	Lean	Obese
Protein	18.5	19.5	19	25	18.5	8
Fat	7.9	27	8	11	10.5	46
Carbohydrates	72.4	53	73	64	71	43

During the post weaning period animals from the same litter were housed together in the same pens. After the animals obtained an average weight of 45 kg or around three months of age, the pigs were transferred to facilities for individual housing and fed a wheat-based HF/HE diet (Table 4). The obese group were fed HF/HE diet *ad libitum* in order to induce obesity while the lean group received 60% of the feed given to *ad libitum* group. The feed was weighed before and after feeding and the pigs were maintained on these diets until they were sacrificed by captive bolt pistol. During the diet-intervention

period, all the animals from experiment II and III were weighed biweekly starting a day prior to switch to experimental feed.

From Exp I, samples were collected when the animals were euthanized. These samples constituted colon with content, terminal ileum with content and cecum with content. In Exp II and III, faecal samples were gathered biweekly and tissue samples from terminal ileum and colon with content were also collected at the end of the study. The samples were immediately frozen in liquid nitrogen and stored at -80° C for later analyses.

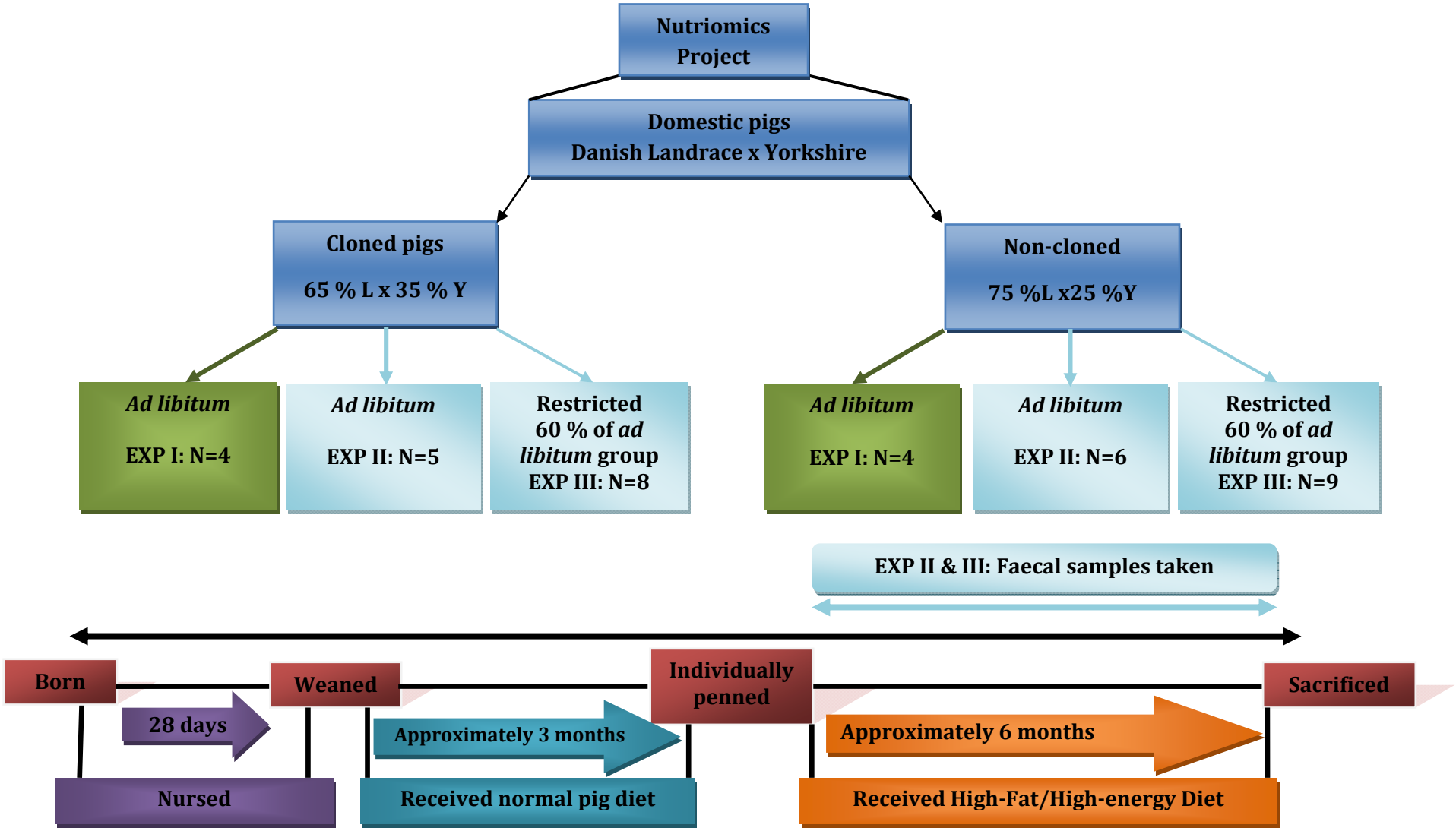

Figure 9 Flow-chart of Nutriomics study.

4.1.2 The Göttingen minipigs Study

As mentioned earlier, due to challenges in obtaining pigs with obese phenotype from Nutriomics project, collaboration was made with groups at University of Copenhagen and NOVO NORDISK A/S. The Göttingen minipigs (N=14) for this study were all female and ovariectomized (Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark). The pigs had been given therapeutic peptides for pharmacological studies prior to the obesity experiments but were subjected to a suitable washout before the start of obesity experiments. At around two years of age, the pigs were housed in facilities provided by the University of Copenhagen (Taastrup, Denmark) until they were euthanized at approximately three years of age. Seven females (n=7) were allocated as the obese group and seven female (n=7) were allocated as the lean control group. The obese pigs were fed Altromin 9033 (mini-pig chow, Christian Petersen A/S, Gentofte, Denmark) with *ad libitum* access to feed and the lean received minipig chow (Altromin 9023) 150 grams, twice a day (Table 4). At the end of the experiment the animals were weighed and the body-fat composition was measured by dual energy x-ray absorptiometry scanning (DXA-scanning) (Hologic Explorer, Santax Medico, Aarhus, Denmark). The Göttingen minipigs were euthanized by pentobarbital and were desangiunated. Colon and cecum samples with content were collected after the animals were euthanized and were frozen in liquid nitrogen and were later stored at -80° C.

4.1.3 The Ossabaw minipigs Study

The samples from female Ossabaw minipigs with polycystic ovary syndrome (metabolic syndrome group) were provided to us by Indiana University-Purdue University Indianapolis Medical Centre and Purdue University breeding colony (West Lafayette, IN, USA). The lean Ossabaw minipigs (n=4) were housed in a group at an average age of six months and received a daily standard chow consisting of 2200 kcal. The animals were provided the chow diet for a period of 10 months until euthanized at an age of 16 months. The obese group of pigs (n=4) were housed individually at an age of six months and were subsequently fed a high-energy atherogenic diet with a daily energy content of 4500-6000 kcal for a period of 8-10 months (Lee et al., 2009b). They were euthanized at the end of feeding experiments at an age of 14-16 months. Overviews of the energy distribution in both groups are mentioned in Table 4. All the pigs were euthanized by cardiectomy by a combination of intramuscular injections of tiletamine-zolazepam (5 mg kg^{-1}) and xylazine (2.2 mg kg^{-1}) while they were under anaesthesia. Samples from colon and terminal ileum including content were collected after the animals were euthanized and were immediately frozen in liquid nitrogen and subsequently stored at -80° C.

4.2 Methodological approaches for studying gut microbiota

Culturing have been one of the oldest approaches for investigating a microbial community but molecular approaches based on 16S rRNA gene such as polymerase chain reaction (PCR) are faster, easier and more precise. Techniques such as, terminal restriction fragment length polymorphism and quantitative real time PCR (qPCR) made it possible to study more complex communities. Species identification of prokaryotes has been done by these methods through identification of 16S rRNA genes. The 16S rRNA gene which consists of nine hypervariable regions that are interspersed with conserved regions is used for characterization of microbial communities. The variable regions of 16S rRNA gene encode structural features that are specific to one or more microorganism and are therefore variant (Van de Peer et al., 1996). The variant regions, V1-V9, of 16S rRNA genes are mainly used for species specific PCR primers for identification of different microbial species (Wang and Qian, 2009). The conserved regions of 16S rRNA gene are mainly involved in essential functions of the bacteria and these conserved regions are present in almost all bacteria. In the field of microbiology conserved or variable regions of 16S rRNA genes are used to design primers which are synthetic oligonucleotides that are complementary to a region in the target deoxyribonucleic acid (DNA). The conserved regions of 16S rRNA gene are used for designing universal primers while variant regions are used to design specific bacterial primers to identify specific bacteria. In the following sections, a brief description of different methods that were used in this thesis will be made.

4.2.1 DNA extraction and purification

One of the important steps before molecular analysis of a biological sample is extraction of DNA from the samples. Most data obtained from human gut microbiota has been through analysis of faecal samples. The collection of the samples are not problematic, however there may be challenges in regard to storage of the samples. Faecal samples taken freshly must be stored immediately at – 80°C to restore DNA and RNA from all the organisms present in the sample which can be challenging for human subjects due to practicalities. Studies have shown radical decrease in the total number of bacteria in faecal samples after less than 24 hours at room temperature (Ott et al., 2004). Therefore it is important to preserve the samples correctly and immediately. In order to avoid highly variable DNA content in each sample, it is also important to store the samples under equal conditions. In this thesis all samples were immediately frozen in liquid nitrogen and later stored at – 80°C. After the samples were moved on ice for DNA extraction purposes, they were than stored at – 20°C. Sampling from all the experiments were performed equally, not only to avoid bias, but to make sure that the obtained results would reflect the actual microbial community in that particular animal.

Several commercial protocols are available for DNA extraction from stool samples. Care must be taken when extracting DNA for microbial studies in order to avoid destruction of several bacterial species by lyses whilst keeping in mind that the cell wall of Gram-positive bacteria are not easily disrupted as opposed to Gram-negative bacteria. Therefore all the DNA extractions for this thesis were performed with an additional step of bead beating in order to mechanically disrupt the rigid cell walls of Gram-positive bacteria. In **Paper I** and **II** the QIAamp DNA Stool Mini Kit was used in combination with a bead beating step which had previously been shown to result in extraction of high quality DNA (McOrist et al., 2002). However this method is very time consuming and has low efficiency in regard to number of samples from which DNA is extracted in a given amount of time. Many commercial automated DNA extracting techniques and kits are available, minimizing the contamination of the samples due to the automated closed system. For **Paper III** a new automated DNA extraction kit, Maxwell system was used. Maxwell system (Promega UK, Southampton, UK) is an automated system that is able to purify DNA in 16 samples in a very short time (~49 min). In a recent comparison study of this system with other DNA purification systems, the authors reported that DNA extraction by Maxwell resulted in high quality and quantity of DNA (Foley et al., 2011). The samples used in **Paper III** were purified by Maxwell system.

After the DNA is extracted, the amount of DNA and the purity can be measured by NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies Inc, USA). Absorbance can measure molecules at specific wavelengths so different molecules absorb the light at different wavelengths. The ratio of absorbance used for assessing DNA purity in a sample is 260/280 (i.e. absorbance at wavelengths 260 and 280 nm) and the accepted ratio for a pure sample is ~ 1.8 for DNA and ~2 is accepted for RNA. Ratios lower than these may indicate presence of contaminants such as proteins and phenols. In **Paper I**, **II** and **III** NanoDrop was used to measure amounts of DNA and verify purity of the extracted DNA.

4.2.2 Polymerase chain reaction (PCR)

PCR was first described by Mullis and co-workers in 1986 (Mullis K. et al., 1986) and became one of the techniques used in the field of microbiology. This technique allowed the exponential amplification of a target DNA, with the subsequent visualization of the target DNA by gel electrophoresis. For this purpose primers complementary to each strand of DNA are designed. PCR consist of three steps performed on automated thermocycler, starting with an initial denaturing step, an annealing step followed by elongation step. During the DNA amplification process, the number of DNA is doubled in each cycle. Many factors can affect the PCR reaction and therefore many parameters such as annealing temperature and magnesium chloride must be optimized for correct and optimal amplification of DNA. In this thesis PCR was performed as described in **Paper I**, **II** and **III**.

4.2.3 Terminal restriction fragment length polymorphism (T-RFLP)

Terminal restriction fragment length polymorphism (T-RFLP) is a molecular fingerprinting method for analysis of a microbial community. A fingerprint of a community is obtained by this method through which the diversity and evenness in complex environmental samples can be analyzed, however identification of bacteria or bacterial groups can be performed through clone libraries (Liu et al., 1997;Leser et al., 2002). In this thesis T-RFLP was used in **Papers I** and **II**. T-RFLP consists of several steps as shown in Figure 10, starting with DNA extraction and purification from samples consisting of a complex microbial community, such as faecal samples, followed by PCR amplification by a fluorescently labelled forward primer at 5'-end of the primer. The obtained PCR products are subsequently purified and digested with a restriction enzyme such as *HhaI*. A 5'- end fluorescently labelled DNA size standard (using energy transfer technology (ET)) is added to the obtained digested and cleaned up PCR products. These digested and labelled PCR products are separated by capillary gel electrophoresis automatically on a gene analyzer and the terminal restriction fragments (T-RFs) are eventually visualized (Liu et al., 1997).

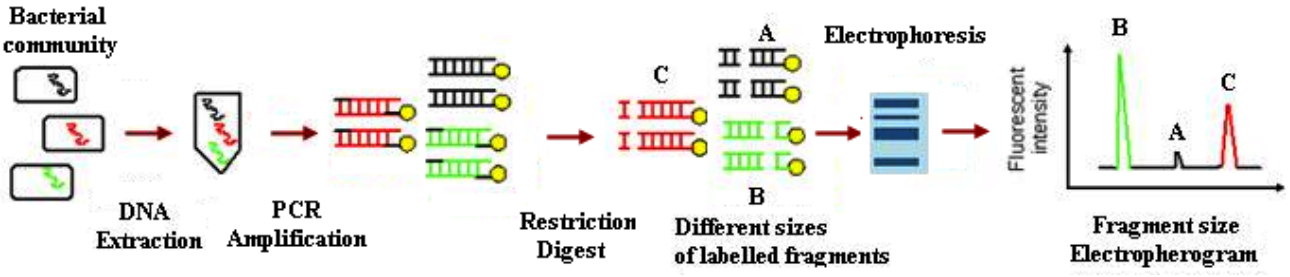

Figure 10 Steps in terminal restriction fragment length polymorphism (T-RFLP). The bacterial DNA from a sample is extracted and the bacterial DNA is amplified and fluorescently label led. The DNA is then digested at different sites by restriction enzymes. The samples are run on gene analyser and the restriction fragments (T-RFs) are visualised. Each top on Electropherogram indicates a bacterial fragment. The x-axes indicates fragment length of the T-RF and the Y-axes indicates intensity of T-RFs

At different sites of 16S rRNA gene there are different restriction sites for different species and thereby through T-RFLP, the obtained T-RFs represent different organisms. The obtained T-RF patterns are visualized as peaks in electropherogram which are then collected by the computer software and can be analyzed by simulated computer software such as BioNumerics. The obtained patterns in peak height and the area are the fluorescent T-RFs, representing the length in base-pairs. Eventually, a comparison of the obtained bands with an internal standard ladder can be made. A detailed description of T-RFLP method, the primers used for analysis of samples and data analysis can be found in **Paper I** and **II**.

4.2.4 Fluorescent in situ hybridization

Fluorescent in situ hybridization (FISH) is one of the widely used techniques in environmental microbiology by which prokaryotic cells can be visualized in their natural environment. This method can be used to identify the presence or absence of bacteria in a given sample through visualization by microscopy. In this method the microbial cell in a given sample is fixed through chemical treatment and the bacterial 16S rRNA gene is subsequently hybridized with labelled oligonucleotide probes. The probes are designed so that they are labelled directly with a fluorochrome and the samples are then analyzed by laser scan microscopy. More specifically, the probes must be able to penetrate through the cells and then bind to 16S rRNA gene. The ribosomal-RNA (rRNA) oligoprobes can be designed with different specificities and these probes can be used to identify each bacteria cell by microscopy in complex environments such as the intestinal microbiota. Techniques such as T-RFLP do not give the exact picture of the microbiota and therefore in combination with other techniques such as FISH, certain strains of bacteria can be visualized and localized in a microbial environment. This technique is very useful if the localization of certain bacteria in a tissue sample is to be investigated, however quantification of prokaryotes in a given sample by counting fluorescent units is not precise Although several studies have used the method to quantify specific bacterial divisions in faecal samples (Duncan et al., 2008;Nadal et al., 2008). For example a certain probiotics strain can be observed visually by FISH if the right probe is chosen. In this thesis probes were designed to target species belonging to *Prevotella* in order to identify this group of bacteria in colon and terminal ileum of cloned and non-cloned pigs, but the probes were never used. However, FISH was performed to visualize the bacteria belonging to two bacterial phyla, *Bacteroidetes* and *Firmicutes* in both cloned and the non-cloned pigs in cross-sections of terminal ileum and colon (with content) but the result obtained by FISH have never been published. The procedures used in this thesis to design *Prevotella* probes and protocols on FISH are found in appendix (Figure 19).

4.2.4 Quantitative real time PCR (qPCR)

Unlike conventional PCR which provides information at the end of the run, through qPCR the relative abundance of bacterial genes in different phylogenetic groups are quantified in real time, i.e. detection at the same time the gene is amplified. There are different qPCR platforms with different assays. In this thesis fluorescent chemistry, SYBR Green, was used for relative quantifications of bacteria in **Paper I** and **II**. In this method the double stranded DNA is first denatured upon heating, followed by annealing of the primers and extension step. SYBR Green binds to all newly synthesized double stranded DNA and fluoresces. This fluorescence is accumulated through the cycling and is measured at the end of each PCR cycle.

The final threshold cycle values (Ct) values obtained are the fluorescent intensity generated by SYBR Green above the back ground fluorescent levels (Figure 11). The Ct values are used to quantify the amount of DNA in each sample.

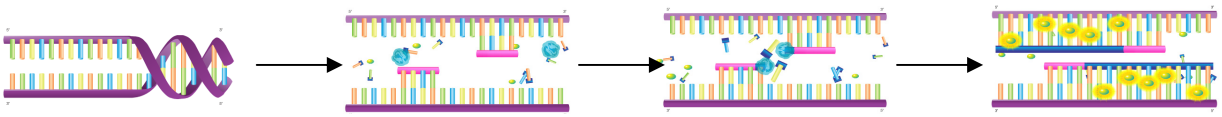

Figure 11 qPCR by SYBR Green chemistry. Denaturing step (94 °C), Annealing (45-65 °C), Extension (65-75 °C) and Elongation

4.2.4.1 Relative quantification of intestinal bacteria by qPCR

In **Paper I** 16S rRNA gene DNA extracted from terminal ileum and distal colon content for quantification of the relative abundance of the phyla *Bacteroidetes* and *Firmicutes.* DNA was extracted from pure culture of three bacteria; *Clostridium perfringens* (NCTC 8449), *Odoribacter splanchnicus* (isolate DJF_B089) and *Escherichia coli* (ATCC 25922) to establish standard curves for each primer set. To further verify if there were any inhibitors present in the samples from colon and terminal ileum, standard curves were obtained from serial dilutions of genomic DNA from two random samples (cloned and non-cloned animals). All Detailed information on primers and conditions are presented in **Paper I** and **II**. After each run on Rotor-Gene Q (Qiagene), a melting curve analysis was performed to confirm specific amplification of the target gene. The data was analyzed by ΔCt method as explained in **Paper I** and **II**.

4.2.5 High-throughput qPCR-based approach in quantification of gut bacteria in pigs

Another qPCR platform was used in order to identify bacterial groups in different phylogenetic levels (**Paper II**). The platform is designed by another Ph.D. student and at the time of this thesis the platform is still undergoing optimizations and therefore the specifics on this platform will not be mentioned in this thesis. In short, the qPCR platform is a 48.48 Dynamic Array Integrated Fluidic Circuits (Fluidigm, CA, USA) which combines 24 primers (in duplicates) with 48 samples to run 2304 simultaneous qPCR reactions. In the platform two universal primers are included and the rest of the primers are specific primers designed to target 16S rRNA gene of different bacterial phylogenetic groups. At Phyla level, it targets *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, *Veruccomicrobia* and *Spirochaetes*, of which neither of the results obtained, were used in this study due to specificity problems. Therefore qPCR (Rotorgene, Qiagene) was additionally performed on *Bacteroidetes* and *Firmicutes* in **Paper II** and **III**. In class level the primers targeted *Bacilli, β-, γ-, δ-, and ε-Proteobacteria*. In family level the primers target *Lactobacillaceae, Streptococcaceae, Cl. Cluster I, Cl. Cluster IV, Cl. Cluster XIV, Bifidobacteriaceae* and *Enterobacteriaceae*. At genus and species level, the

primes targeted *Enterococcus, Bacteroides, Cl, perfringens* and *E-coli*, respectively. In family level, Genus and species level most of the results were used for presentation in **Paper II**. As this method has just been designed and not published at the time of this thesis was due, much information about this method is not provided. For more information on this platform it is referred to data by (Hermann-Bank *et al*. 2012, *in prep*). Figure 12 shows a heatmap obtained from one of the chip-runs which was successful, however as it is shown in the figure many samples were not detected.

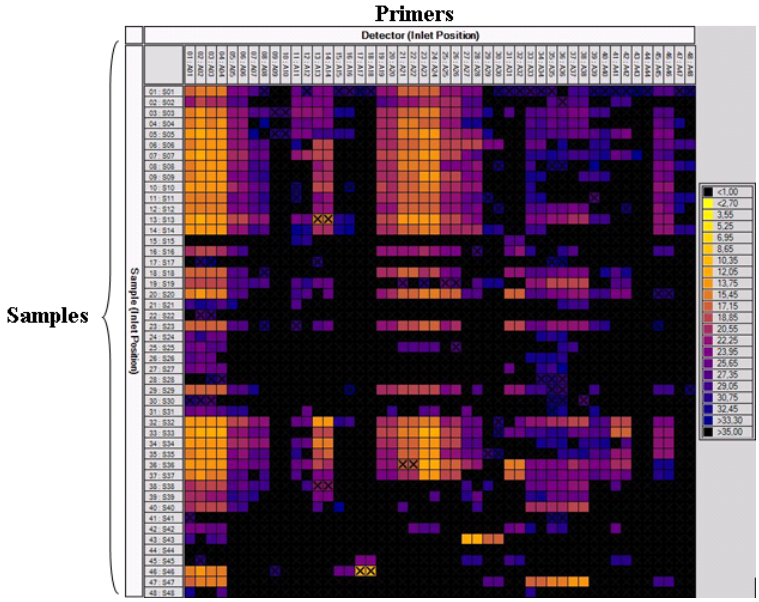

Figure 12 Micro Fluidic Dynamic Array chip.

Recently a new high throughput qPCR platform was developed (Bergström et al., 2012) in which 31 primer sets targeting bacterial 16S rRNA gene at different phylogenetic was evaluated. In this method the efficiency of each amplicon group was calculated and used to determine initial concentration of each specific 16S rRNA gene and subsequently all the initial concentrations for target bacteria (specific 16S rRNA gene) were normalized against the initial concentration of universal bacteria in the same platform (Bergström et al., 2012). This made the quantification of fold-changes or fold-differences between specific groups of bacteria possible. The Fluidigm qPCR platform used in this thesis did not provide these concentrations and therefore the data was analyzed by several different methods and the only method that could provide some information other than Ct values obtained was the Livak method which is generally used for gene expression analysis (**Paper I and II**). Through this method fold-change/fold-differences were calculated between groups was calculated.

4.2.5.1 Fluidigm data analysis

Due to the fact that this method was used at its' early stages, information regarding details on the optimizations of the chip and many other factors were not provided by the author of the paper prior to publication of her paper. In this thesis, this method was merely used to verify some of the findings that had already been established by other methods such as deep sequencing in **Paper III.** In **Paper II**, fluidigm chip was used to relatively quantify, the relative abundances of some bacterial groups that were designed in this chip. The analysis of the data was performed by several methods as mentioned before and finally through literary research and consultations by others who performed RT-qPCR, Livak method was chosen for analysis (Livak and Schmittgen, 2001). This method is primarily used for gene expression data whereby relative gene expression can be analyzed and not the absolute gene expression. The assumption is that target and reference genes are amplified with 100% efficiencies and within 5% of each other's efficiencies. In this method there is a need for a calibrator or a gene that the rest of the genes are calibrated against. One of the important factors in using this method is to verify amplification efficiency of both target and reference gene so that their amplification efficiency is near 100%. In this study the lean pigs in each group and study were considered as the "Calibrator", while the obese pigs were the "test". The "target gene" was the amplified DNA by specific primer, for example "*Firmicutes"*, while "reference gene" was considered the amplified DNA by universal primer "GB-B" in case of this chip. Here the Ct values are used to calculate ΔCt followed by calculation of ΔΔCt and $2^{-\Delta\Delta Ct}$. There are three steps to the Livak method: first the normalization of the Ct value of target gene to Ct value of reference gene, secondly normalization of ΔCt of test sample to ΔCt of calibrator and finally the expression ratio or here the fold differences are calculated. For more detail on the calculations it is referred to appendix (Table 9).

4.2.6 Microbial community in cloned and non-cloned pigs

In January 2009 only samples from eight nutriomics pigs were available. These samples constituted of colon and terminal ileum with content. The microbiota from terminal ileum and colon of four obese cloned pigs (Exp I) and four non-cloned pigs (controls) (Exp I) were analyzed by T-RFLP. Furthermore, FISH was performed on unwashed tissue samples from cloned and non cloned pigs in Exp I. These were preliminary studies and the results obtained from T-RFLP and fluorescent in situ hybridization were not included in any paper. In the subsequent experiment, Exp II, it was decided to gather faecal samples biweekly from the five cloned and six non-cloned obese pigs (Figure 9). The faecal samples that were gathered biweekly were analyzed by T-RFLP and qPCR. These results are presented in **Paper I**. Animals from Exp II were the lean control group for the obese group. There were eight lean cloned pigs and nine non-cloned pigs in this group. Faecal samples were gathered biweekly, but unfortunately some samples were missing throughout the period in different groups, therefore

samples were analyzed from faecal samples available monthly. T-RFLP was performed on these samples. Furthermore the samples from colon and terminal ileum of all the animals, from experiment I and II were analyzed by qPCR and the new qPCR platform (Fluidigm). These results are presented in **Paper II**.

4.2.7 Deep sequencing by Illumina

Next generation sequencing is a powerful tool for characterization of microbial communities. The 16S rRNA gene is often used for taxonomic classification and characterization of these microbial communities. The two sequencing technologies 454 (Roche) and Illumina are currently the most frequently used for this purpose. In general, the 454 technology allows sequencing of products of up to about 500 bases, whereas the maximal length of sequencing is restricted to about 100 bp with the Illumina (Degnan and Ochman, 2012). On the other hand the sequencing depth (amount of sequences) using Illumina is much higher than with the 454. The Illumia HiSeq 2000 platform uses bridge amplification, i.e. the DNA fragments that are to be sequenced are attached to a glass slide by binding to DNA adaptors on the slide. There are three steps in the sequencing process by Illumina; first a sequence library must be generated, followed by cluster generation and finally sequencing. The three steps in Illumina sequencing are described briefly in the next sections.

4.2.7.1 Library generation

In the sequence library generation the ends of the DNA fragments to be sequenced are linked to adapters. The adaptor oligos are necessary for the amplification and sequencing. DNA fragments longer than approximately 100-300 base pairs are sheared to shorter fragments before ligation to the adapters (Figure 13 (1)).

4.2.7.2 Cluster generation

In the second step, each end of the single adaptor labelled DNA molecule is initially attached to a flow cell by hybridizing to fragments on the flow cell that are complementary to the sequence of the adapters. This creates a so-called bridged of the DNA fragments that should be sequenced. PCR of these molecules creates millions of copies of each strand leading to generation of clusters. After the cluster formation, the reverse strands are cleaved and removed by a washing step creating single-stranded DNA fragments anchored to the surface of flow cell. The sequencing primer is hybridized to the adaptor sequence which was previously ligated to the DNA fragment (Figure 13 (2)).

4.2.7.3 Sequencing

The clusters are sequenced on the genome analyser by a technique called cyclic reversible termination, where the extension by polymerase is performed with reversible terminators. DNA template is sequenced one base at a time by reversible terminators which are four fluorescently labelled nucleotides, deoxynucleotides that are able to reversibly terminate. Here, as the blocking group is terminated, the polymerase incorporates one labelled nucleotide at a time.

The nucleotides that failed to incorporate to the DNA are washed away. After each round of DNA synthesis the clusters are excited by a laser beam. This enables each added base to be identified through their colour Figure 13 (3).

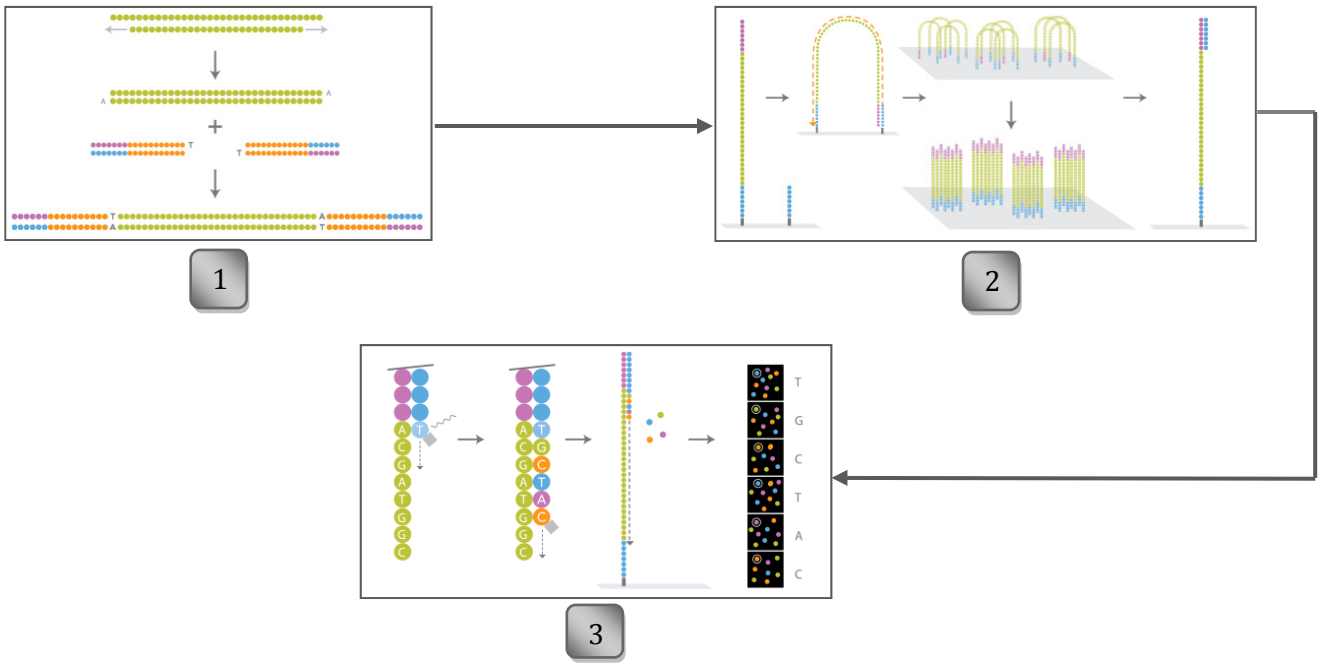

Figure 13 Illumina sequencing steps. Step 1: Library generation, 2: Cluster generation, 3: sequencing. (Adapted from http://www.dkfz.de/gpcf/illumina_hiseq_technology.html)

4.2.7.3.1 Sequencing data analysis

Obtained data were analysed using the BION-meta software (Larsen et al., 2012). The taxonomic classification of sequences was done according to the Greengene database. Initially the sequences were de-multiplexed. De-multiplexing was performed according to the primer, the barcode sequences and was than cleaned at both ends. This was done by removal of bases that had a quality less than 96%. The identical sequences were eventually clustered and aligned into consensus sequences with a setting of 99.8% base quality. The consensus sequences that had at least 30 nucleotides were mapped into a table according to the individual barcodes.

The obtained consensus sequences were taxonomically classified against the Greengene SSU database using a word length of 8 and a match minimum of 30%. The top one percent of the obtained hits from the Greengene database was then used for taxonomical classification of the consensus sequences. In the final step the number of reads for each barcode was normalized. This was done for making valid statistical analysis that can be performed directly between individuals in each experiment.

4.2.8 General statistical analysis

Non-parametric approaches are made when data is not normally distributed. For comparing and finding the differences between two groups in non-parametric test, Mann-Whitney U test (Mann and Whitney, 1947)can be used (**Paper I**, **II** and **III**). Correlation analysis as performed in **Paper I** and **II** can be performed by Spearman Rank order correlation which is a non-parametric approach for correlation analysis. However multivariate analysis such as principal component analysis (PCA) can be used to investigate the interaction between different variables and PCA analysis was used in **Paper I** and **II**. From the normalized reads the bacterial diversity analysis was performed by Shannon-Weaver index of diversity ($H' = -\sum pi \ln(pi)$) (Hill, 1973). Most of the data was analyzed in GraphPad prism version 5.00 for Windows (GraphPad software, San Diego CA USA).The obtained sequences from Illumina was normalized by Log transformation prior to analysis and data were analyzed using one-way ANOVA or two-way ANOVA when appropriate with post hoc tests; Kruskal Wallis (McKight and Najab, 2010)and Bonferroni's post hoc tests (Cleophas and Zwinderman, 2011), and the P-values < 0.05 were considered significant. These data are presented in **Paper III**.

4.2.9 Characterization of gut microbiota in Ossabaw and Göttingen minipigs

Samples from terminal ileum and colon with content from Ossabaw minipigs were taken in USA, at Indiana University of Purdu. The DNA was extracted from the following samples: lean colon digesta (N=4), lean terminal ileum digesta (N=2), obese with MetS colon digesta (N=3) and obese terminal ileum digesta (N=3). The amounts and purity of DNA was measured by Nanodrop as described before. The obtained DNAs were subsequently PCR amplified with bar coded primers. The quality and quantity of PCR products were assessed by Agilent 2100 Bioanalyzer which is a microfluidic-based platform to quantify and measure the quality of DNA, using the Agilent DNA 1000 kit (Agilent Technologies, Waldbronn, Germany). Each PCR product fragment was measured and non-template control samples were run on Agilent to verify for contamination. Finally the concentration of the DNA samples were equilibrated against the concentration of the sample with the lowest DNA concentration and the samples were subsequently pulled to one sample. The sample containing bar coded DNA from all the extracted DNA samples, was purified by using Qiagen MiniElute PCR purification kit (Qiagen GmbH, Hilden, Germany) by which any base pairs under 70 bp are removed in order to remove PCR artifacts. The samples were then sequenced and the sequencing was conducted on the Illumina HiSeq

2000 platform as described before (Illumina, Inc. San Diego, California, 92122 USA). The DNA was submitted to the National High-throughput DNA Sequencing Centre at Copenhagen University, Denmark, for sequencing on an Illumina HiSeq™ 2000 platform.

5 General results

5.1 Result from Nutriomics project

As described in the previous chapters, the samples obtained from the first set of cloned pigs were terminal ileum, cecum and colon all with content. The cloned pigs (*n*=4) from Exp I of Nutriomics study weighed 152.3 ±8.1kg and non-cloned controls (*n*=4) weighed 156.1 ±5.3 kg. T-RFLP was per formed on DNA extracted from all of the mentioned samples and data were analysed. FISH was performed on the cross section of terminal ileum and colon, using specific probes for *Bacteroidetes* and *Firmicutes*. The analysis of microbial community in terminal ileum and colon was performed by qPCR and high-throughput qPCR (Fluidigm) and are presented in **Paper II**. The T-RFLP data that was not included in any **Papers** are therefore presented here.

In all the experiments (Exp I, II, II), the cloned and non-cloned pigs had different phenotypes according to several blood parameters as presented by (Christensen et al., 2012)and these differences were mainly in lactate and cholesterol (P<0.001). However no difference was observed in glucose or insulin levels between lean and obese cloned or non-cloned pigs. The most phenotypic significant difference between lean and obese animals was their weight as presented in Table 5. The baseline weight between the lean and obese pigs was significantly different from the lean (restricted diet group) cloned and non-cloned pigs. Furthermore the weight of cloned (*n*=4) and non-cloned pigs (*n*=4) from Exp I at baseline was not provided.

Table 5 Weight of cloned and non-cloned pigs before the diet intervention study (Baseline) and at the end of diet intervention (Endpoint) in both lean (restricted diet group) and obese (*ad libitum*) cloned and non-cloned pigs, (Mean±SEM)

	Cloned L x Y Pigs			Non-cloned L x Y		
	Lean (*n*=9)	Obese (*n*=10)	P-value	Lean (*n*=8)	Obese (*n*=9)	P-value
Baseline weight (kg)	65.1 (±7.4)	38 (±4.1)	<0.009*	61.7 (±2.3)	38 (±2.3)	<0.0001
Endpoint weight (kg)	127.1 (±5.9)	147.5 (±5.9)	<0.02	119.1 (±3.2)	170.1 (±5.4)	<0.0001

5.1.1 Experiment I: Gut microbiota of obese cloned and non-cloned pigs: (unpublished data)

The colonic and ileal microbiota in four obese cloned and four obese non-cloned pigs from Exp I were investigated by T-RFLP. Only the T-RFs between 60 and 800 bp were analyzed and comparisons of differences between cloned and non-cloned pigs' overall microbial communities at endpoint was investigated. The most predominant terminal restriction fragments (T-RFs) (bacterial load) larger than 1% revealed that the lean cloned pigs and non-cloned pigs had a different overall composition of their colonic microbiota (Figure 14), however this was not significant. One particular T-RF (218 bp) higher than all the other T-RFs in the control pigs but this was not significant (P<0.4). The subsequent

T-RFs were analyzed *in siloco* by using the MICA online softawas (Shyu et al., 2007) and 16S rRNA gene sequences were assigned to their taxonomic names on the Ribosomal Database project (Maidak et al., 2001). The T-RF 218 may represent bacteria belonging to the phylum *Firmicutes*. Several T-RFs' with mean relative abundance of more than one percent were present in cloned pigs and absent in non-cloned pigs.

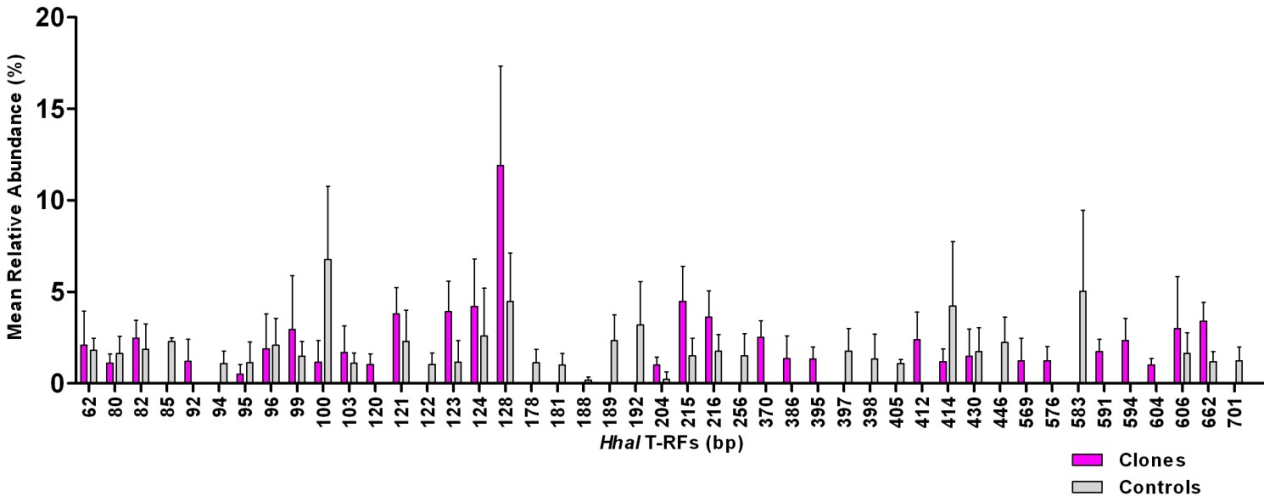

Figure 14 Mean relative abundance of the most predominant T-RFs (>1%) in the colon samples at endpoint in cloned (clones) and non-cloned pigs (controls). The error bars represent standard deviation.

The Shannon-Weaver index of diversity was used to estimate the diversity of the bacterial fragments based on all of the initial T-RFs and comparisons of cloned and non-cloned pigs with respect to this index showed that controls had a slightly higher diversity than clones both in colon and terminal ileum (Figure 15), although this was not significant (Figure 15). Furthermore, the bacterial diversity between colon and terminal ileum was not different in cloned or non-cloned pigs.

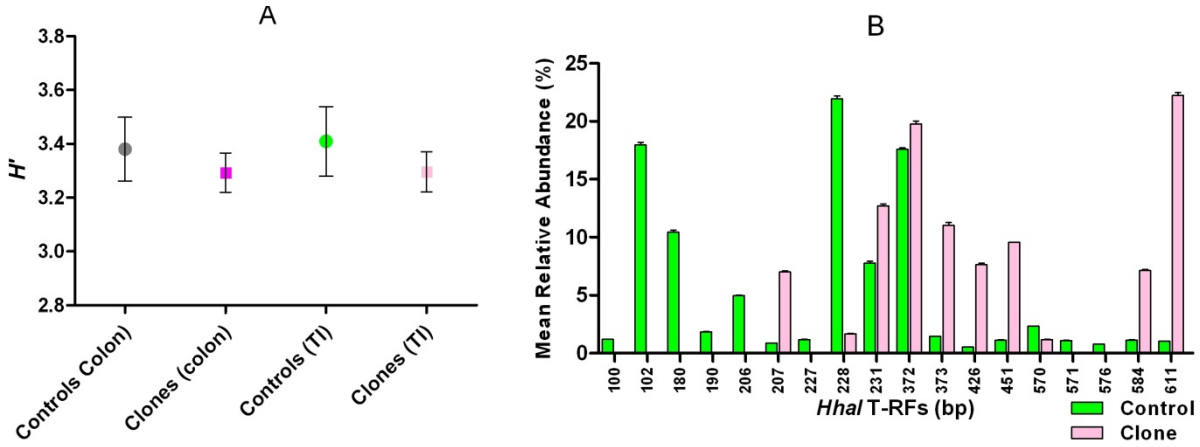

Figure 15 Average diversity of colon and terminal ileum microbiota in each group of animals (A) Mean relative abundance of the most predominant T-RFs (>1%) in the colon samples at endpoint in clones and controls (B). The bacterial diversity was calculated based on T-RFs (bp) at endpoint in colon of controls and clones as calculated by Shannon Weaver diversity index. Results are presented in mean and the error bars represent standard deviations.

The microbiota of terminal ileum and cecum in the four obese cloned pigs and the four obese non-cloned pigs were investigated by T-RFLP. The data showed that the numbers of T-RFs larger than 1% in terminal ileum and cecum were much lower than what was found in colon, while there were more T-RFs in cecum samples than terminal ileum. In all the samples (colon, ileum and cecum), one T-RF, 584 bp, in cloned pigs was larger than non-cloned pigs.

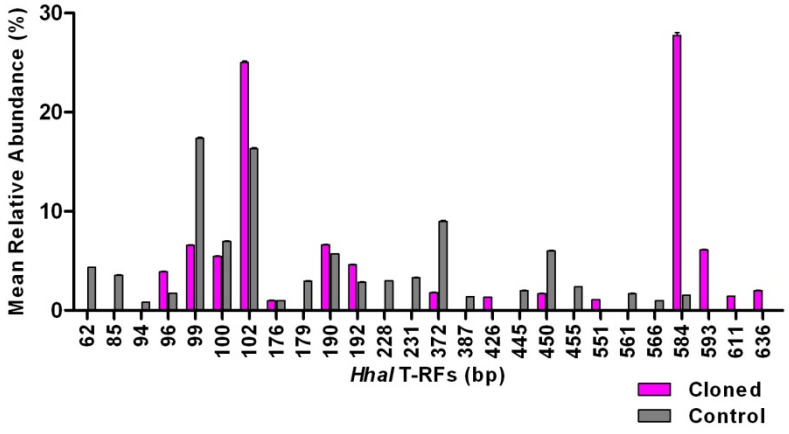

Figure 16 Mean relative abundance of the most predominant T-RFs (>1%) in the cecum samples at endpoint in cloned (clones) and non-cloned pigs (controls). The error bars represent standard deviation.

Some of the T-RFs were higher in cloned pigs as compared to controls (584 and 611) and these were analyzed *in silico* in MICA. The matched bacterial fragments to bp 584 and 611 may belong to phyla *Firmicutes*. However *in silico* analysis of T-RF bp is not precise and therefore no conclusion was made from these data.

The same purified DNA samples used in this study were later analysed by qPCR and Fluidigm. These results are presented in **Paper II**.

5.1.2 Experiment II: Gut microbiota of obese cloned and non-cloned pigs (Paper I)

In this study the faecal microbiota of the obese cloned (*n*=5) and non-cloned pigs (*n*=6) from Exp II (Figure 9) was analyzed and the results are presented in **Paper I**. Overall, the composition of the gut microbiota was not different between cloned pigs and the non-cloned pigs in Exp II. Based on T-RF profiles the cloned pigs' microbial community was neither more similar to that of non-cloned pigs, nor more divers. Furthermore, the bacterial diversity in faecal microbiota of clones and controls in Exp II did not change over the course of obesity from before the start of diet-intervention studies and at the diet-intervention study. In both cloned and non-cloned pigs the total numbers of T-RFs were increased from baseline to endpoint however the overall T-RF profiles were not significantly different between cloned and non-cloned pigs.

5.1.3 Experiment II: *Bacteroidetes* and *Firmicutes* in faecal microbiota (Paper I)

The analysis of specific phyla of bacteria that previously had been reported to be related to obesity (Ley et al., 2005), the *Firmicutes* and *Bacteroidetes,* revealed that the relative abundance of *Bacteroidetes* from baseline (lean state) to endpoint (obese state) was not different (**Paper I**). Interestingly, a negative correlation was observed between weight-gain and relative abundance of *Bacteroidetes* in the cloned pigs during the course of diet-intervention. However at some point, the relative abundance of *Bacteroidetes* began to increase again but never recovered the initial values observed at baseline. The same reaction to obesity was observed in non-cloned pigs but the relative abundance of *Bacteroidetes* was eventually the same as the baseline values. However, a positive correlation between weight-gain and relative abundances of *Firmicutes* was observed in both obese cloned and non-cloned pigs.

5.1.4 Experiment III: Gut microbiota of lean cloned and non-cloned pigs (Paper II)

A profile of faecal microbiota of cloned and non-cloned pigs on restricted diet (the lean group) was obtained by T-RFLP. The PCA analysis of most predominant T-RFs (>1%) revealed that the lean cloned pigs and non cloned pigs had a different overall composition of their faecal microbiota throughout the study but mainly at endpoint. The PCA analysis of the raw data obtained from T-RF intensities by BioNumerics revealed separate clustering of cloned and non-cloned pigs (Figure 17). However this data was not presented in any paper as these only represent the bacterial load and intensities, which may fluctuate depending on many factors.

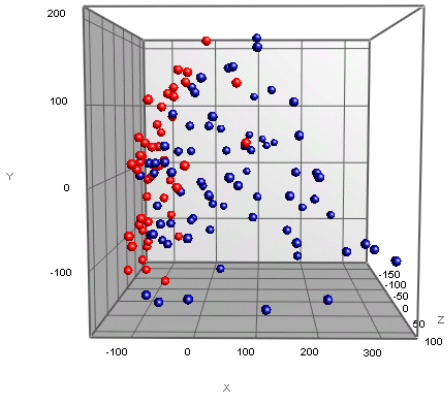

Figure 17. PCA analysis of all the T-RFs (intensities) as obtained by Bionumerics. The first component is the intensity and shows the difference in T-RFs or bacterial load. Cloned pigs (●), Non-cloned pigs (●)

The bacterial diversity in faecal microbiota over the course of diet intervention study and also at baseline (start of diet intervention studies) and endpoint (end of diet intervention studies) were estimated by Shannon-Weaver index. The results showed similar bacterial diversity between lean cloned and non-cloned pigs at both sampling times. Furthermore no change in the diversity of bacteria in faecal microbiota was observed over time. The overall microbial composition according to T-RFs was neither different between cloned and non-cloned pigs, nor was there a significant difference between T-RFs at baseline and end-pint. However, in non-cloned pigs, at endpoint one T-RF (584 bp) was significantly higher than all the other T-RFs. These results are presented in **Paper II**.

5.1.5 Experiment II: Gut microbiota of lean and obese cloned and non-cloned pigs (Paper II)

In **Paper II** the relative abundance of *Firmicutes* and *Bacteroidetes* in colon and terminal ileum microbiota of cloned (n=17) and non-cloned pigs (n=19) on restricted and *ad libitum* HF/HE diet was performed by qPCR. The microbiota in colon of lean cloned pigs revealed a significantly higher abundance of *Firmicutes* and a lower abundance of *Bacteroidetes* than the obese cloned pigs. The relative abundance of *Firmicutes* in colon of non-cloned pigs was also higher in lean pigs than in obese non-cloned pigs. However no difference was observed in relative abundance of *Bacteroidetes* between lean and obese non-cloned pigs. Furthermore a clear higher abundance of *Bacteroidetes* was observed in colon of both obese and lean pigs as compared to terminal ileum. Briefly, there were differences in relative abundance of *Bacteroidetes* and *Firmicutes* between lean and obese pigs' intestinal microbiota.

The qPCR by Fluidigm showed several differences between lean and obese pigs, both in the cloned group and non-cloned group of pigs. **Paper II** provides a detailed description of the differences observed. Briefly, in the lean cloned pigs, colon microbiota there was a higher relative abundance of *Lactobacillaceae* than in colon of lean non-cloned pigs. In obese non-cloned pigs the relative

abundance of *Lactobacillaceae* in colon was significantly higher than the lean non-cloned pigs, which implicates that the lean non-cloned pigs had low abundances of *Lactobacillaceae*.

5.2 The gut microbiota in Ossabaw and Göttingen minipigs (Paper III)

The phenotypic characteristics of lean and obese Ossabaw minipigs were different in body weight (P=0.01), and blood parameters such as triglycerides (P<0.008) and total blood cholesterol (P=0.03). In Göttingen minipigs there was a significant difference between weight of lean and obese (P<0.001) and one blood parameter, fasting insulin (P=0.01). The fasting insulin was very high in lean Göttingen minipigs s compared to obese Göttingen minipigs (Table 6).

Table 6 Blood parameters and weight in Ossaabw and Göttingen minipigs. Data from Ossabaw minipigs is adopted from(Lee et al., 2009b) and the data from Göttingen minipigs is adopted from (Moesgaard et al, *In prepn*) (Mean±SEM).

	Ossabaw minipigs			Göttingen minipigs		
	Lean (*n*=4)	Obese (*n*=4)	P-value	Lean (*n*=7)	Obese (*n*=7)	P-value
Body weight (kg)	60.4 (±6.6)	98.3 (±2.9)	0.01	49.8 (±1.6)	90.1 (± 4)	<0.001
Triglycerides (mmol/l)	0.3 (±0.03)	0.5 (±0.03)	0.008	0.3 (±0.04)	0.5 (±0.07)	0.07
Total cholesterol (mmol/l)	2.0 (±0.1)	12.9 (±2.0)	0.03	1.8 (±0.1)	2.0 (±0.1)	0.2
Fasting blood glucose (mmol/l)	4.2 (±0.1)	4.6 (±0.2)	0.1	3.8 (±0.1)	3.7 (±0.4)	0.9
Fasting insulin (pmol/l)	15 (±3.5)	34.7 (±7.5)	0.1	141.2 (±32.4)	39.2 (±9.9)	0.01

In **Paper III** the intestinal microbiota of Ossabaw minipigs was characterized by Illumina sequencing. However these results are based on low number of animals in each group (n=4) and conclusion from these results must be made accordingly. Several differences were observed between lean and obese Ossabaw minipigs in different taxonomic levels. Furthermore, there were differences in the abundance of bacteria between colon and ileum with significantly lower abundances of *Bacteroidetes* in tetminal ileum than colon in both lean and obese Ossabaw minipigs (Table 7) (**Paper III**).

Table 7 The most abundant bacteria in different taxonomic levels. Abundance of bacteria are presented as percent mean relative abundance in colon and terminal ileum (TI) microbiota of Göttingen and Ossabaw mini pigs.

	Colon		Cecum		Colon		TI		
	Obese	Lean	Obese	Lean	Obese	Lean	Obese	Lean	
Phyla (%)	**Göttingen**				**Ossabaw**				**Phyla (%)**
Firmicutes	47.7	51.7	45.1	52.8	45.7	35.8	63.6	55.9	**Firmicutes**
Bacteroidetes	37.0	37.6	42.2	36.7	35.9	48.7	17.4	22.6	**Bacteroidetes**
Spirochaetes	7.0	5.0	6.7	5.5	5.4	4.1	11.4	13.2	**Proteobacteria**
Tenericutes	5.4	3.3	3.9	2.6	6.0	6.5	2.1	3.1	**Spirochaetes**
Proteobacteria	0.8	0.6	0.6	0.8	4.1	3.0	2.1	2.1	**Fusobacteria**
Actinobacteria	0.3	0.3	0.3	0.4	0.9	0.5	1.9	0.8	**Actinobacteria**
Fibrobacters	0.3	0.3	0.2	0.5	0.4	0.2	0.4	1.5	**Tenericutes**
Other	1.4	1.3	1.1	1,1	1.6	1.2	1.1	0.8	**Other**
Class (%)	Obese	Lean	Obese	Lean	Obese	Lean	Obese	Lean	**Class (%)**
Clostridia	44.8	48.6	42.3	49.9	40.2	29.4	53.5	38.7	**Clostridia**
Bacteroidia	36.8	37.4	42	36.6	35.7	48.5	15.9	22.5	**Bacteroidia**
Spirochaetes	7	5	6.7	5.5	5.5	6.4	11.0	17.2	**Bacilli**
Mollicutes	4.3	2.7	3.4	2.3	3.7	2.6	9.6	11.5	**γ-Proteobacteria**
Bacilli	2.9	3	2.7	2.9	6.0	6.5	2.1	3,1	**Spirochaetes**
Erysipelotrichi	0.96	0.5	0.5	0.3	4.1	3.0	2.1	2,1	**Fusobacteria**
Methanobacteria	0.7	0.3	0.2	0.3	0.9	0.6	1.9	1.0	**β-Proteobacteria**
Actinobacteria	0.3	0.4	0.3	0.4	0.9	0.5	1.5	0.8	**Actinobacteria**
Fibrobacteres	0.3	0.3	0.3	0.3	0.9	0.4	0.4	0.3	**Synergistia**
β-Proteobacteria	0.2	0.2	0.2	0.5	0.2	0.2	0.2	1.3	**Mollicutes**
α-Proteobacteria	0.2	0.2	0.3	0.2	0.3	0.2	0.3	0.3	**ε-Proteobacteria**
WPS-2	0.1	0.2	0.1	0.1	0.2	0.4	0.2	0.2	**α-Proteobacteria**
Other	1.4	1.2	1	0.7	1.4	1.3	1.3	1	**Other**
Family	Obese	Lean	Obese	Lean	Obese	Lean	Obese	Lean	**Family**
(Bacteroidales)	22.4	21.4	21.7	19.5	19.4	30.8	8.1	12,4	***Prevotellaceae***
Ruminococcaceae	16.6	18.5	15.2	17.9	11.0	5.0	17.7	11.5	***Clostridiaceae***
Lachnospiraceae	15.9	15.7	15.2	19.2	9.2	9.1	11.5	9.8	***Lachnospiraceae***
Prevotellaceae	10.6	11.9	15.1	13.0	5.6	4.2	13.7	6.5	**(Clostridiales)**
(Clostridiales)	7.0	7.7	6.8	7.0	9.5	7.7	6.3	6.8	***Ruminococcaceae***
Spirochaetaceae	6.5	4.7	6.3	5.2	8.6	9.6	4.0	5.6	**(Bacteroidales)**
Porphyromonadaceae	2.9	3.0	3.8	2.9	3.2	4.6	4.3	13.9	***Lactobacillaceae***
Veillonellaceae	1.9	2.9	2.1	2.1	6.0	6.5	2.1	3.0	***Spirochaetaceae***
Anaeroplasmataceae	3.1	1.8	2.5	1.6	2.3	1.7	6.7	3.4	***Enterobacteriaceae***
Clostridiaceae	1.7	2.0	1.6	2.1	4.6	4.5	3.1	2.4	***Bacteroidaceae***
Other	11.4	10.4	9.7	9.5	20.6	16.3	22.5	24.7	**Other**
Genus	Obese	Lean	Obese	Lean	Obese	Lean	Obese	Lean	**Genus**
(Bacteroidales)	22.4	21.4	21.7	19.5	18.4	28.2	7.6	11.5	***Prevotella***
(Ruminococcaceae)	10.7	12.5	9.7	11.6	10.9	4.9	17.5	11.4	***Clostridium***
(Lachnospiraceae)	9.3	9.2	8.6	10.9	5.6	4.2	13.7	6.5	**(Clostridiales)**
Prevotella	7.9	9.0	11.4	9.4	8.6	9.6	4.0	5.6	**(Bacteroidales)**
(Clostridiales)	7.0	7.7	6.8	7.0	3.2	4.6	4.3	13.9	***Lactobacillus***
Treponema	6.4	4.7	6.3	5.2	5.9	4.2	3.8	4.0	**(Ruminococcaceae)**
(Prevotellaceae)	2.7	3.0	3.7	3.6	6.0	6.5	2.1	3.0	***Treponema***
Ruminococcus	2.9	3.0	2.9	3.5	4.6	4.5	3.1	2.4	***Bacteroides***
Coprococcus	1.9	1.8	1.8	2.4	3.3	3.6	3.2	3.1	**(Lachnospiraceae)**
Clostridium	1.6	2.0	1.5	2.1	1.4	1.1	3.8	2.7	***Epulopiscium***
Lactobacillus	1.5	1.2	1.5	1.4	3.1	1.9	1.5	1.6	***Fusobacterium***
Paludibacter	1,2	1,3	1,7	1,3	1.1	0.8	3.2	1.6	**(Enterobacteriaceae)**
Roseburia	1,2	1,2	1,4	1,6	1.5	1.7	1.2	1.3	***Ruminococcus***
Blautia	1,2	1,3	1,1	1,6	1.8	2.0	0.8	1.0	***Parabacteroides***
Other	22.1	20.7	19.9	18.9	24.6	22.2	30.2	30.4	**Other**

The analysis of colon and cecum microbiota of Göttingen minipigs revealed that the five most abundant phyla constituted 98.1% of all the obtained phyla consisted of *Firmicutes*, *Bacteroidetes*, *Spirochaetes*, *Tenericutes* and *Proteobacteria* with *Firmicutes* having the highest abundance in cecal

microbiota of lean pigs. The results obtained by Illumina sequencing on bacterial abundance in colon and cecum microbiota of Göttingen minipigs are presented in **Paper III**. Table 7 gives an overview of the abundance of bacteria in different taxonomic groups in cecum and colon of Göttingen mini pigs.

The diversity analysis by Shannon-Weaver performed on sequencing data obtained from genus, showed no difference between lean and obese colon microbiotal diversity. However in Ossabaw minipigs there seemed to be a higher diversity in obese minipigs but this was not significant (Figure 18).

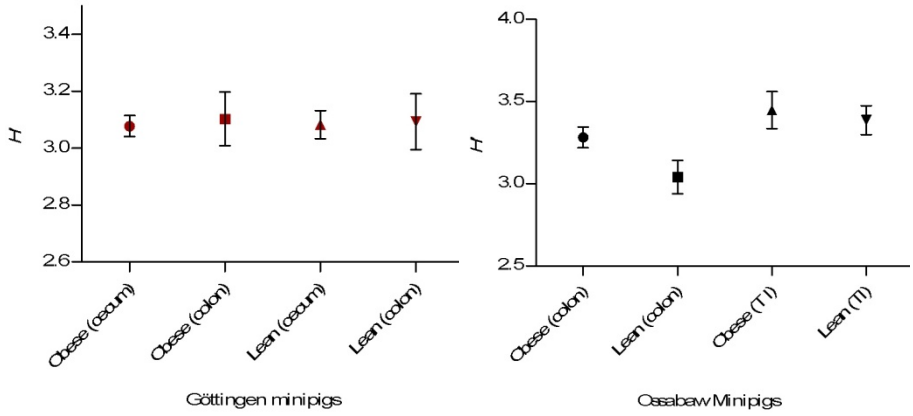

Figure 18 Bacterial diversity from genus level by Shannon weaver diversity (*H'*) in Göttingen (colon and cecum) and Ossabaw minipigs (colon and terminal ileum)

The obtained data from qPCR analysis (Fluidigm) of Göttingen and Ossabaw minipigs'gut microbiotas were not conclusive due to result obtained from the chip which for the most was non-detectable. The results that were than analysed showed fold differences between lean and obese Göttingen minipigs with a 7.6 fold higher abundance of Cl. cluster XIV family in the colon of obese Göttingen minipigs as compared to the lean group ($P < 0.0001$).

6 Papers

Paper I

Pedersen R., Andersen D., Mølbak L., Stagsted J., Boye M. "**Changes in the gut microbiota of cloned and non-cloned control pigs during development of obesity".** 2012 *Accepted in BMC Microbiology*

In this paper the composition of faecal microbiota was investigated by qPCR and T-RFLP in cloned and non-cloned pigs during a high-fat/high-energy feeding period of 136 days of *ad libitum* feeding.

The most intriguing finding was the continual increase in proportions of *Firmicutes* in the faecal microbiota during the diet-intervention period. There was a negative correlation with decrease in *Bacteridetes* and weight-gain in cloned pigs. However, this was not observed in the non-cloned pigs, in which the relative abundance of *Bacteroidetes* fell and stabilized again so that there was no significant difference from before the diet-intervention study and at the end of the study. T-RFLP results revealed no differences between cloned and non-cloned pigs in regard to inter-individual variation.

Main findings: HF/HE diet-induced obesity caused changes in the gut microbiota of cloned and non-cloned pigs. The cloned pigs did not show a reduced biological variation as compared to non-cloned pigs.

Running head: Gut microbiota during development of obesity in cloned pigs

Changes in the gut microbiota of cloned and non-cloned control pigs during development of obesity

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Key words: Gut microbiota, Cloned pigs, diet-induced obesity, Bacterial diversity, *Bacteroidetes*, *Firmicutes*

ABSTRACT

Background: Obesity induced by a high-caloric diet has previously been associated with changes in the gut microbiota in mice and in humans. In this study, pigs were cloned to minimize genetic and biological variation among the animals with the aim of developing a controlled metabolomic model suitable for a diet-intervention study. Cloning of pigs may be an attractive way to reduce genetic influences when investigating the effect of diet and obesity on different physiological sites. The aim of this study was to assess and compare the changes in the composition of the gut microbiota of cloned vs. non-cloned pigs during development of obesity by a high-fat/high-caloric diet. Furthermore, we investigated the association between diet-induced obesity and the relative abundance of the phyla *Firmicutes* and *Bacteroidetes* in the fecal-microbiota. The fecal microbiota from obese cloned (n = 5) and non-cloned control pigs (n= 6) was investigated biweekly over a period of 136 days, by terminal restriction fragment length polymorphism (T-RFLP) and quantitative real time PCR (qPCR).

Results: A positive correlation was observed between body-weight at endpoint and percent body-fat in cloned ($r=0.9$, $P<0.0001$) and in non-cloned control pigs ($r=0.9$, $P<0.0001$). Shannon Weaver and principal component analysis (PCA) of the terminal restriction fragments (T-RFs) revealed no differences in the bacterial composition or variability of the fecal microbiota between the cloned pigs or between cloned and non-cloned control pigs. Body-weight correlated positively with the relative abundance of *Firmicutes* in both cloned ($r=0.37$; $P<0.02$) and non cloned-control pigs ($r=0.45$; $P<0.006$), and negatively with the abundance of Bacteroidetes in cloned pigs ($r=-0.33$, $P<0.04$), but not in the non-cloned control pigs.

Conclusion: The cloned pigs did not have reduced inter-individual variation as compared to non-cloned pigs in regard to their gut microbiota in neither the obese nor the lean state. Diet-induced obesity was associated with an increase in the relative abundance of *Firmicutes* over time. Our results suggest that cloned pigs are not a more suitable animal model for gut microbiota-obesity related studies than non-cloned pigs. This study is the first to evaluate if cloned pigs than conventional pigs in diet-intervention, obesity and gut microbiota research

BACKGROUND

Obesity and its associated morbidities have become an increasing problem in many countries around the world. While traditionally regarded as primarily a question of a sedentary lifestyle in which energy intake

exceeds energy expenditure, new studies also point to the composition of the intestinal microbiota as a potentially contributing factor. In studies of diet induced obesity and its association with the gut microbiota, it may be preferable to eliminate the influence of host genotype on the composition of the gut microbiota by choosing genetically identical animals. Some early investigations comparing the composition of the microbiota in human mono-zygotic twins (MZ) with di-zygotic twins (DZ) reported that the host genome was influencing the microbial composition in the gut [1,2]. A similar study based on 16S rRNA gene analysis indicated that bacterial community in human MZ twins was slightly more similar than in unrelated individuals [3] suggesting that genetically identical individuals harbour a similar gut microbiota. In a more recent study on the relationship between gut microbiota, diet and genetic influences in mice, the authors stated that the changes in gut microbiota were unrelated to genetically induced obesity and were merely due to high-fat (HF) diet [4]. Therefore, the influence of the host genome on the gut microbiota currently remains controversial.

When choosing an animal model for studying human diseases, it is important to choose animals that physiologically resemble humans. Pigs are good models for humans, primarily due to close resemblance of their anatomy and physiology of the digestive system and because pigs are omnivorous like humans [5,6]. Consequently, pigs are widely used in studies of human lifestyle-related diseases such as diabetes, cardiovascular disease and metabolic syndrome [7,8]. Using cloned pigs in obesity-related studies could provide a more homogenous experimental model, hence the cloning in this study was performed to minimize genetic influences and thereby reduce inter-individual variation [9].

One of the main focuses of obesity-related gut microbial studies have been to identify groups of bacteria that are correlated with the obese state, and initially the relative abundance of *Bacteroidetes* and *Firmicutes* in the gut microbiota was linked to obesity. In pigs, as in humans [10] and other mammals [11], the two main phyla of bacteria in the gut microbiota are *Bacteroidetes* and *Firmicutes* [12,13]. Previous studies have reported a greater proportion of Firmicutes in obese mice [14] when compared with their leaner counterparts and a reduced ratio of *Firmicutes* to *Bacteroidetes* in a small group of obese humans on a weight loss regimen [15]. A similar result in a study of lean and obese pigs revealed a negative correlation between percentage of *Bacteroidetes* and body-weight [16]. Furthermore, a fluorescence in situ hybridization (FISH)-based study on obese adolescents during weight loss regimens showed a decrease in the phylum *Firmicutes* [17]. However

several studies suggest a decrease in ratio of *Firmicutes* to *Bacteroidetes* in obese and overweight subjects [18] and suggest diet to be a contributing factor in shaping the gut microbial community and not the bacterial proportions [19,20]. Other observations in humans, suggest obesity to be associated with a lower bacterial diversity [3], while other studies showed no difference in the abundance of bacteria in the gut microbiota between lean and obese individuals that were on weight maintaining diet [21]. Hence this putative relationship between obesity, diet and specific phyla of bacteria in the gut microbiota is still controversial and there are few studies on the association between the gut microbiota and obesity during the development of obesity. Therefore, the focus of this paper was to investigate the gut microbiota in cloned pigs compared with non-cloned control pigs and to further elucidate if diet-induced obesity over time is associated with changes in the gut microbiota. We hypothesized that the composition of the gut microbiota would be more similar among the cloned pigs compared to non-cloned controls. The second hypothesis was that weight-gain would be related to an increase in the ratio of *Firmicutes* to *Bacteroidetes* as well as a decrease in the diversity of the gut microbiota. We therefore investigated the changes in the gut microbiota of cloned and control pigs beginning with lean pigs during a period of 136 days on a high-fat/high-caloric (HF/high-caloric) diet.

METHODS

Animals

The animals for this experiment were pigs of similar genotype of Danish Landrace and Yorkshire. Six female siblings from a normal litter (the control group) (75 % Landrace x 25 % Yorkshire) were obtained after standard artificial insemination followed by caesarian section. The cloning experiments were performed using donor cells obtained from a 65 % Landrace x 35 % Yorkshire sow as described previously [9]. The cloned embryos were then transferred surgically to surrogate sows (recipients) five to six days after cloning [9]. Two surrogate sows gave birth to five live female clones by caesarean section. Pigs were reared in the experimental stables at University of Aarhus (Tjele, Denmark). All the experimental animal studies were approved by the Danish Animal Experimental Committee.

Experimental set up and sample collection

The pigs in the experiment were weaned at 28 days of age and subsequently fed a standard pig-diet with an energy distribution of 18.5 % protein, 7.9 % fat, 72.4 % carbohydrate and 1.2 % fibre, for approximately 61

days. During this post weaning period animals from the same litter were housed together in the same stable. At 96 days (cloned pigs) and 89 days (non-cloned controls) of age (baseline), the pigs were transferred to facilities for individual housing and fed a wheat-based HF/high-caloric diet consisting of 19.5 % protein, 27 % fat, 53 % carbohydrates and 0.5 % fibre [22] with *ad libitum* access to the feed in order to induce obesity. The feed was weighed before and after feeding and the pigs were maintained on this diet for a period of 136 days until they were euthanized. The cloned and non-cloned control pigs were weighed biweekly starting a day prior to switch to HF/high-caloric feed and the body-fat composition of the animals was measured by computer tomography (CT) scan at the end of the experiment. During this period, fresh feces collected biweekly were snap-frozen in liquid nitrogen and stored at -20°C until later analyses.

Terminal restriction fragment length polymorphism (T-RFLP)

The fecal microbiota from all the pigs were analyzed by terminal restriction fragment length polymorphism (T-RFLP) fingerprint profiles as described previously [23]. In brief, DNA was extracted from 200 mg feces by using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions, with an additional step of bead beating in order to disrupt the cell wall of Gram-positive bacteria. The concentrations of DNA were measured in each sample by a spectrophotometer and adjusted to 5 ng µl-1 (NanoDrop Technologies,Wilmington, DE, USA). Amplification of 16S rRNA gene DNA were performed in duplicates by using 16S rRNA gene DNA bacterial specific primers, Eub-8fm (5'-AGAGTTTGATCMTGGCTCAG- 3') labelled with 5´FAM and Eub-926r (5-'CCGTCAATTCCTTTRAGTTT- 3') (DNA Technology, Aarhus, Denmark) [23]. Each PCR mix contained 5 µl of 10x Fermentas Taq-buffer, 4 µl MgCl2, 2.0 µl deoxyribonucleotide triphosphate (dNTP), 0.5 µl Fermentas Taq-polymerase, 0.5 µl of each primer and 35.5 µl nuclease-free water and 5 ng µl-1 DNA (final concentration of 0.2 ng). The cycling conditions were: initial denaturation at 94 0C for 6 minutes (min) followed by 32 cycles of denaturing at 940 C for 45 seconds (s), annealing at 560 C for 45 s, an extension step at 720 C for 2 min, and a final extension at 720 C for 10 min. The PCR products were subsequently verified by gel electrophoresis and purified by High Pure PCR Purification Kit (Roche Applied Sciences, Mannheim, Germany). The purified PCR product (200 ng) was digested with 2.0 µl of the restriction enzyme HhaI (Promega Corporation, Madison, USA) at 37°C for 3 h. Two µl of the digested PCR products, 10 µl

formamide and 0.50 µl Megabase ET900-R Size Standard (GE Health Care, Buckinghamshire, UK) were mixed and run in duplicates on a capillary electrophoresis genetic analyzer (Genetic Analyzer 3130/3130xl, Applied Biosystems, Carlsberg, CA). The terminal restriction fragments (T-RFs), representing bacterial fragments in base pair (bp), were obtained and the analysis of T-RF profiles and alignment of T-RFs against an internal standard was performed using the BioNumerics software version 4.5 (Applied Maths, Kortrijk, Belgium).

T-RF fragments (range of 60-800 bp) with a difference less than two base pairs were considered identical. Only bands present in both duplicates were accepted as bacterial fragments from which the duplicate with the best intensity was chosen for microbial profiling. The obtained intensities of all T-RFs were imported into Microsoft Excel, and all intensities below 50 were removed. In each sample, the relative intensity of any given T-RF was calculated by dividing the intensity of the T-RF with the total intensity of all T-RFs in the sample. The most predominant T-RFs with a mean relative intensity above one percent were selected for all further analyses and procedures (except calculation of the diversity and similarity) and their identity was predicted in silico, performed in the MiCA on-line software [24] and Ribosomal Database Project Classifier (322.864 Good Quality, >1200) [25].

T-RFLP statistical analysis

All T-RFs between 60 and 800 bp were imported into the statistical software programs Stata 11.0 (StataCorp, College Station, TX), Unscrambler version 9.8 (CAMO, Oslo, Norway) and Microsoft Excel sheet were used for further analyses. Principal component analysis (PCA) was used to explore group differences in the overall microbial communities both for comparisons between cloned pigs and non-cloned controls at the different sampling points and to investigate if samples from pigs with the largest weight gain during the study period clustered together, irrespective of their genetic background. The latter was also investigated by relating the whole microbial community to the weight gain at the different sampling points, involving all predominant T-RFs simultaneously in the models. For this purpose partial least square regression (PLS-R) was used, which is a supervised model, meaning in this case that the variation in the weight (gain) data is used to actively decompose the variation in the bacterial data. In both analyses, the T-RFs were standardized (centred and

1/SD) prior to the modelling phase to ensure that all of them would equally influence the models, and possible outliers were inspected visually and with *Hotelling* T^2.

The diversity index was calculated as described previously [26]. In brief, the Shannon-Weaver index of diversity (*H'*) based on all of the initial T-RFs was used to determine the diversity of the bacterial fragments. Group comparisons of the diversity index in cloned versus non-cloned controls were calculated at each of the sampling points. As the Shannon-Weaver index was not normally distributed Mann Whitney U test and Spearman correlation were applied. The *H'* values are represented in figures as mean and error bars representing standard deviations (SD). Dice similarity between groups based on all the T-RFs were calculated in BioNumerics (Applied Maths, Kortrijk, Belgium) and the results are presented as mean values. T-RFs in the figures are presented as mean and standard error of the mean (SEM). A significant difference was considered when P-value was less than 0.05 ($P<0.05$).

Faecal samples and bacterial strains for qPCR

The extracted DNA from the faecal samples used for the T-RFLP analyses were analyzed by qPCR as well but only samples taken monthly were chosen for qPCR analysis. Three bacterial strains (*Clostridium perfringens* (NCTC 8449), *Odoribacter splanchnicus* (isolate DJF_B089) and *Escherichia coli* (ATCC 25922), representing the *Firmicutes* and *Bacteroidetes* phyla and general bacteria, respectively and six randomly chosen extracted DNA samples (divided equally into clones and controls) were used to optimize the PCR conditions.

qPCR primers and conditions

The 16S rRNA gene DNA primers for *Bacteroidetes* and Firmicutes used in this study were designed by Baccetti De Gregoris et al. [27] and conditions optimized for the thermocycler used (Rotor-Gene Q Real Time PCR cycler (Qiagene)). The universal primer used in this study had an amplicon length of 147 bp (S-D-Bact-0907-a-S-20 5'-AAACTCAAAGGAATTGACGG-3'; S-D-Bact-1054-a-A-20 5-' ACGAGCTGACGACAGCCATG-3') [12]. The specific primer sets for *Bacteroidetes* (798cfbF 5' CRAACAGGATTAGATACCCT'3 and cfb967R 5' GGTAAGGTTCCTCGCGTAT '3) and Firmicutes (928F-Firm 5' TGAAACTYAAAGGAATTGACG '3; 1040firmR, 5' ACCATGCACCACCTGTC '3) had an

amplicon length of 240 bp and 200 bp, respectively [27]. All qPCR reactions contained 12.5 µl of SYBR® Green JumpStart™ Taq ReadyMix™ without MgCl2 (Sigma-Aldrich, Copenhagen, Denmark), 0.3 µmol l-1 of each primer and 5 µl of template DNA adjusted to 5 ng µl-1. MgCl2 optimization was performed and a final concentration of 2.5 mM MgCl2 was chosen. The annealing temperature was optimized by using 16S rRNA gene DNA extracted from fecal samples and DNA extracted from different bacteria. Subsequently, all the primers and other PCR conditions were verified by conventional PCR and gel electrophoresis. A non template control (NTC) was included in each run. qPCR was performed with an initial denaturing step of 10 min at 95°C, 95°C for 30 s, 35 cycles of 56°C for 20 s and an elongation step of 72°C for 20 s. A melting curve analysis was performed after each run to detect any primer-dimers in each sample. The threshold cycle (Ct) and calculated concentrations (copies µl-1) were determined automatically by the Rotor Gene software (Rotor-Gene Q 2.0.2 (Qiagene)).

Analysis of data from qPCR

qPCR was performed to quantify relative abundance of the phyla *Bacteroidetes* and *Firmicutes,* respectively, present in each sample. The measured bacterial copy numbers of the 16S rRNA gene from bacteria belonging to the phylum *Bacteroidetes* and the phylum *Firmicutes* was calculated against 16S rRNA genes obtained from all bacteria and the percentage of copy numbers from the two phyla in each sample was subsequently calculated and statistically evaluated by Mann Whitney U test. Further correlation analyses were performed using Spearman correlation coefficient and $P<0.05$ was generally considered statistically significant. A standard curve was constructed for specific and universal primer sets and assays using tenfold serial dilutions of the extracted DNA from *C. perfringens*, *O. splanchnicus* and *E. coli* all DNA samples in the range 2.5 $x10^{2}$ ng μL^{-1} to $2.5x10^{-6}$ ng μL^{-1}. Furthermore, serial dilutions corresponding to the previously described dilutions of genomic DNA from two random samples were used to construct standard curves, to further verify if PCR inhibitors were present in extracted DNA from faecal samples.

RESULTS

Weight of the animals

At baseline, just before the animals were transferred to the *ad libitum* high-fat (HF)/high-caloric diet, the cloned (96 days old) and non-cloned control (89 days old) pigs weighed 38 ± 4.1 kg and 37.9 ± 2.3 kg,

respectively. Daily weight-gain in cloned pigs (n=5) was 0.78 ± 0.04 kg and in control pig (n=6) 1.05 ± 0.03 kg, corresponding to a lower daily feed intake by cloned pigs than the controls. The clones weighed 143.6 ± 8.8 kg as opposed to the control pigs 179.5 ± 4.0 kg at the time they were euthanized (endpoint) and consequently the controls weighed significantly more than the clones (35.9 kg) ($P<0.004$) at the end of the study. CT scanning of body-fat showed that on average the obese non-cloned control pigs had a higher percentage of body-fat (41.1 ±1.3 kg) than the obese cloned pigs (28.4 ± 2.3 kg) ($P<0.004$). There was a positive correlation between body-fat composition and body weight at the end of the diet-intervention study in non-cloned control pigs ($r=0.9$, $P<0.0001$) as well as in cloned pigs ($r=0.9$, $P<0.0001$) (Figure 1).

The compositional diversity of the gut microbiota

The PCA analysis of the overall composition of the gut microbiota in all animals did not reveal separate clustering of the T-RF profiles between the cloned pigs and the non-cloned controls. To test if the gut microbiota between cloned pigs was more similar than between non-cloned control pigs, a dice similarity score was calculated showing that the microbiota in cloned pigs was neither more uniform within the group nor more diverse compared to non-cloned control pigs (Figure 2A). Furthermore, there was no difference in Shannon-Weaver index between cloned and non-cloned control pigs at the start of diet-intervention (baseline) with Shannon index (*H'*), *H'*=2.6 (2.3-2.8) and *H'*=1.7 (1.5-2.8), respectively. Within the control group a slight increase ($P<0.01$) in the diversity of the gut microbiota was observed from baseline to end of diet-intervention (endpoint) (*H'*=3, 2.3-3.4), while no difference was observed in the cloned pig group (*H'*=3.3, 2.3-3.4) (Figure 2B). Furthermore, there was no correlation between diversity of microbial community as found by Shannon Weaver index and gain in weight (Figure 2B).

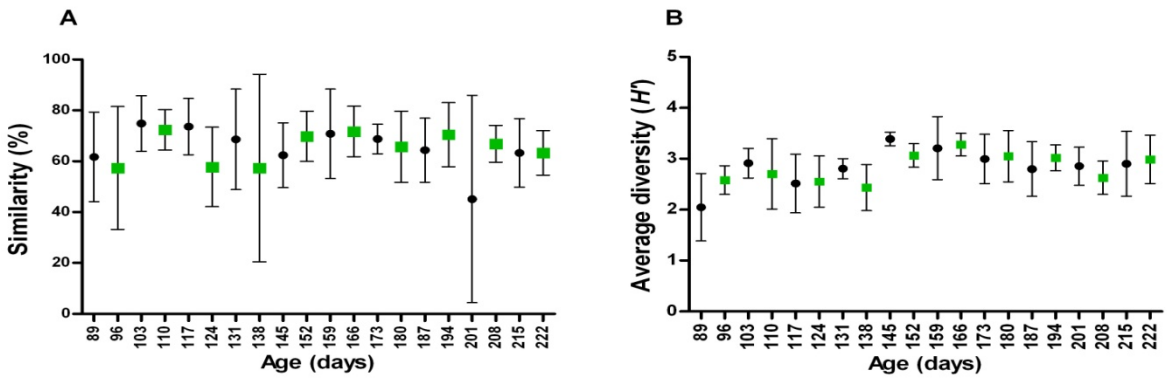

Figure 2 Similarity (A) and diversity (B) of gut microbiota. The similarity and diversity was calculated based on T-RFs (bp) at different age interval in non-cloned control pigs (●) and cloned pigs (■) by Dice similarity index and Shannon Weaver index. Results are presented in mean and the error bars represent standard deviations (SD).

The bacterial load (including all initial T-RFs between 60 and 800 bp) in the faecal microbiota of cloned pigs and non-cloned control pigs was similar throughout the intervention period, P<0.08 and P<0.3, at baseline and endpoint, respectively. In general, the T-RF profiles were similar in the cloned pigs and non-cloned pigs (Figure 3A and B). Both cloned pigs and non-cloned control pigs had 11 T-RFs with a relative abundance larger than one-percent in common at baseline and 17 T-RFs at endpoint (Figure 3A and B) but the difference in T-RFs between the cloned pigs and non-cloned control pigs was not significant (P<0.08).

In the non-cloned control group, one individual T-RF with a length of 102 bp was found higher at baseline compared to endpoint (P<0.04) (Figure 3A) and within the cloned pig group one T-RF (93 bp) was higher at endpoint than at baseline (P<0.01) (Figure 3B). At baseline in the non-cloned control group, the relative abundance of T-RF 93 bp was less than one percent and a significant increase in T-RF 93bp from baseline to endpoint (P<0.005) was observed. The *in silico* analysis of the obtained T-RFs indicated that the T-RF 93 bp and T-RF 102 bp may be bacterial fragment belonging to the phylum *Bacteroidetes*.

In the cloned pigs 28 out of the 47 most predominant T-RFs with an intensity of more than 1% represented 92% of the all the bacterial fragments present at baseline (Figure 3A). In non-cloned control pigs, 18 out of 42 bacterial fragments constituted 96 % of T-RFs at baseline. At the endpoint in cloned pigs, 26 T-RFs constituted 83% of all 85 bacterial fragments and thereby reflecting more bacterial richness as compared to baseline (42 original bacterial fragments). In controls 22 out of 82 bacterial fragments represented 82% of all bacteria in faecal samples (Figure 3A and B), indicating an increase in the total number of T-RFs from

baseline to endpoint. The differences in T-RFs observed between cloned and non-cloned control pigs were not significant.

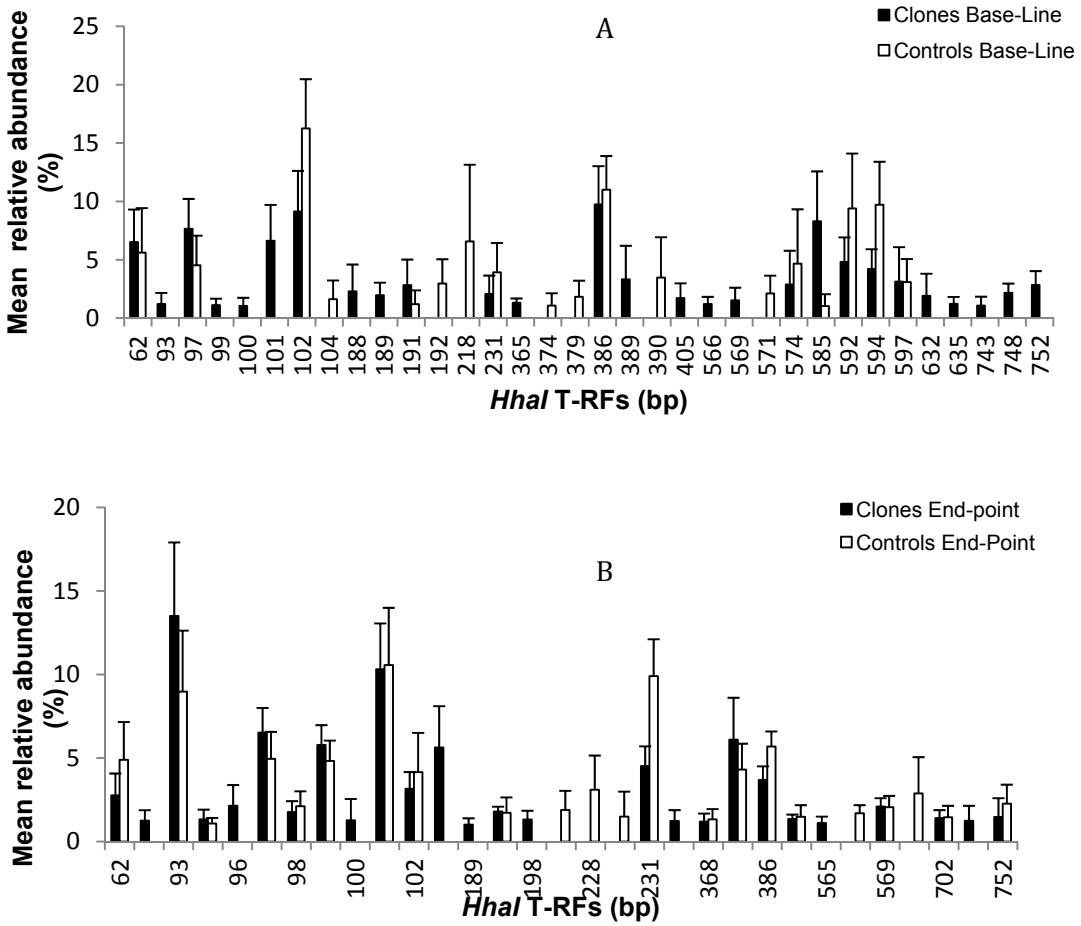

Figure 3 The abundance of bacteria at baseline and endpoint. Mean relative abundance of the most predominant T-RFs (>1%, bp) in the faecal samples at baseline (A) and endpoint (B) in cloned pigs (■) and non-cloned control pigs (□). The error bars represent standard error of the mean (SEM).

Proportion of Firmicutes and *Bacteroidetes* in the gut microbiota by qPCR

There was no difference in the relative abundance of *Bacteroidetes* between cloned pigs and non-cloned control pig at baseline (P<0.1) or at endpoint (P<0.9) and the same was observed for *Firmicutes* (baseline, P<0.8; endpoint, P<0.7).

At baseline, the average relative abundance of *Bacteroidetes* in cloned pigs was 39% (±4) and in non-cloned control pigs 31% (±3). At endpoint, the average relative abundance of *Bacteroidetes* in the cloned pigs was 38.8 % ±1.4 and non-cloned control pigs 38.6% (±6). There was a negative correlation between weight-gain and relative abundance of *Bacteroidetes* in the cloned pigs (r= -0.33, P<0.04) (Figure 4A). In the cloned pig group a continuous and significant decrease (P<0.008) was observed in phylum *Bacteroidetes* from baseline

and throughout the weight gain (118.9 ±3.2 kg) period up to an age of 208 days (Figure 4A) which then began to rise again until the animals were euthanized. In the non-cloned control pigs, there was a fall in the relative abundance of *Bacteroidetes* starting from age 89 days (weight: 37.9 ± 2.3 kg) until the age of 146 days (weight: 95.5 ±3.9 kg) from which point the relative abundance of *Bacteroidetes* began to increase, meaning that at age 225 days the relative abundance of *Bacteroidetes* was not different between baseline and endpoint (Figure 4B).

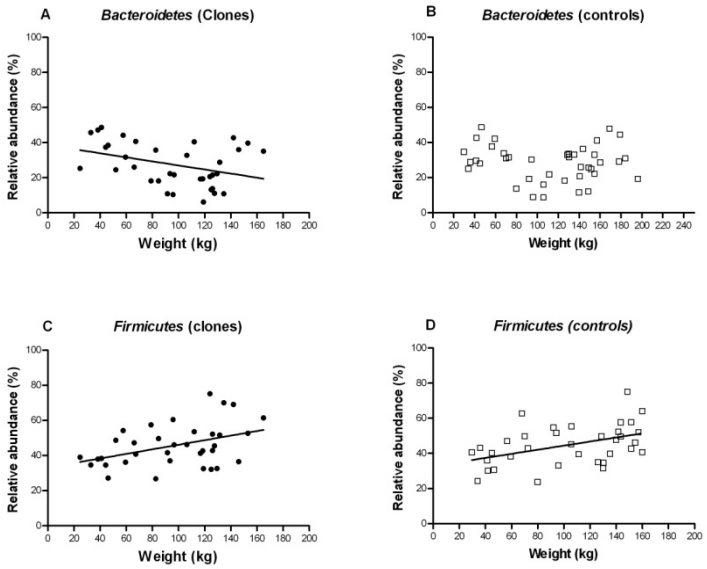

Figure 4 Correlation between weight gain and relative abundance of *Bacteroidetes* and *Firmicutes*. Correlation between gain in weight and relative abundance of *Bacteroidetes* as calculated by Spearman correlation in cloned pigs (A) (r= -0.33, P<0.04) and non-cloned control pigs (B) and correlation between weight-gain and relative abundance of *Firmicutes* in cloned pigs (C) (r= 0.37, P<0.02) and non-cloned control pigs (D) (r=0.45, p<0.006).

In cloned pigs at baseline samples (age 96 days) and samples at age 223 days, there was a significant difference (P<0.01) in relative abundance of *Bacteroidetes* between the two sampling times. This was not observed in the non-cloned control pig group. An increase in percent *Firmicutes* was observed in cloned pigs from baseline (35% ±2), to endpoint (54.7% ±5.4; P<0.009) (Figure 4C) and the same was observed in non-cloned control pigs from baseline (37.7% ± 3) to endpoint (57% ±2.2) (P<0.0001) (Figure 4D). This positive correlation between the weight gain and the percentage of *Firmicutes* during the study period was observed both in cloned pigs (r= 0.37, P<0.02) and non-cloned control pigs (r=0.45, p<0.006).

DISCUSSION

In order to establish a better understanding of the underlying causes of obesity and the effect of obesity on different body sites, the cloned pigs and non-cloned control pigs employed for our study were also

investigated in regard to their immunological [28], metabolomics [22] and phenotypic characters [9]. In this study, we investigated the gut microbiota of both cloned and non-cloned control pigs by T-RFLP and found that the gut microbiota within a group of five obese clones was neither more similar nor more diverse than the microbiota within a group of six obese non-cloned control pigs of the same sex and genetic background. The metabolomic phenotyping [9] of these obese cloned and non-cloned control pigs showed that the phenotype of the cloned pigs was different from the phenotype of non-cloned control pigs [9] and that the inter-individual variation amongst these cloned pigs was not less than the inter-individual variation of the non-cloned control pigs that were siblings [22]. Hence, based on these and the findings presented in the current paper it would appear that the cloned pigs do not have identical phenotypes or less inter-individual variation than conventional non-cloned pigs. One explanation for these results could be that in cloning by somatic cell nuclear transfer the animals inherit maternal mitochondrial DNA and even though they have the same somatic DNA, the cloned pigs possess altering phenotypes due to the maternal mitochondrial DNA effect [9]. This raises the question of whether cloned animals are more suitable animal models than conventional non-cloned animals.

The heritable component of an individual and its effect on the microbial community have been investigated before in several human studies; in particular MZ twins have been investigated to minimize the genetic influence in order to get a better understanding of the role of obesity on gut microbiota [3]. When designing an experimental model for gut microbiota related studies, it is important to remove the large variability in the microbial community across individuals, making it necessary to use larger number of animals for valid statistical analysis and interpretation. Therefore, cloned animals could have the potential of becoming good models, by reducing the number of animals needed for an experimental study and providing a less variable population, however, more optimization is needed to improve the quality of the cloned animals.

In regard to obesity related gut microbiota, we did not observe any association between weight gain and change in bacterial diversity, although there was more bacterial richness of in obese pigs. Taken together; these results point to specific changes in the bacterial community over time in both the cloned and non-cloned control pigs.

To get a better profile of the gut microbial community in relation to obesity, we compared the relative abundance of the phyla *Bacteroidetes* and *Firmicutes* in the pigs from baseline and throughout the diet intervention period until endpoint. In the case of *Firmicutes*, we observed an increase in relative abundance of this phylum from baseline to end point, in both cloned and non-cloned pigs and found a positive correlation with *Firmicutes* and weight-gain. This increase in the abundance of the phylum *Firmicutes* with increase in weight is in agreement with observations made in other studies [15]. One study points to a connection between alterations in energy intake and changes in gut microbiota such as increase in abundance of *Firmicutes* [29]. Jumpertz and colleagues [21] found that a 20 % increase in abundance of *Firmicutes* resulted in an increase in energy harvest corresponding to approximately 150 kilo calories. This suggests that the bloom in bacteria belonging to the phylum *Firmicutes* contributes to promotion of obesity and maintenance of the obese state.

The relative abundance of *Bacteroidetes* in the cloned pigs decreased continuously through the diet intervention period but then began steadily to increase until the animals were euthanized. The same was observed in the non-cloned control pig group and eventually the relative abundance of *Bacteroidetes* at end point was not different from baseline. This was unexpected, as previously it has been shown that obese subjects have less *Bacteroidetes* compared to their leaner counterparts [10,16,30]. Furthermore, one study on humans under a weight loss regiment showed [15] an increase in *Bacteroidetes*. One explanation to the observations made in our study could be that the bacteria belonging to phylum *Bacteroidetes* somehow adapt to the HF/high-caloric diet and their number at end point eventually reaches the values observed at baseline. Hildebrandt et al [29] demonstrated a decrease in *Bacteroidetes* and an increase in *Firmicutes* in the gut microbiota of mice independent of obesity but in relation to HF diet in mice [29], while other studies point to the association of HF diet and the changes in abundance of Firmicutes in mice [4]. Together, these studies suggest that the changes in gut microbiota could be due to the HF/high caloric diet and not the state of obesity. Even though we found a positive relation between weight gain and changes in the relative abundance of Firmicutes, we cannot exclude the possibility that the changes were also in relation to HF/high-caloric. Therefore, the gut microbiota could be a potential therapeutic target to fight obesity.

CONCLUSION

Here we conclude that cloned pigs do not appear to have smaller inter-individual variation as compared to the sibling non-cloned pigs with regard to their gut microbiota, and because it is both time consuming and costly, they are not more suitable than conventional pigs for gut-microbiota-obesity related studies.

Our findings agree with the hypothesis that the diet-induced obesity is related to changes in the relative abundance of Firmicutes and *Bacteroidetes* and especially an increase in proportion of the bacteria belonging to the phyla Firmicutes. We also point to HF/high-caloric diet as a contributing factor that changes the gut microbial community. To our knowledge this is the first study that has investigated the effects of diet-induced obesity on gut-microbiota in cloned pigs. More investigation is needed to optimize the cloning of experimental animals which could eventually offer a more controlled experimental model.

COMPETING INTEREST

All authors declare no financial or any other competing interest.

AUTHORS' CONTRIBUTIONS

MB, LM and RP designed the study experiments. RP carried out the experimental work, data and statistical analysis and wrote the manuscript. A.D.A performed the statistical analysis on T-RFLP Shannon-Weaver diversity and PCA and contributed to writing of the manuscript. JS designed and conducted the animal and the diet-intervention experiments. All authors read, corrected and approved the final manuscript.

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Paper II

Pedersen R., Andersen AD., Stagsted J., Boye M.: **"The gut microbial composition of lean and obese cloned and non-cloned pigs on high-energy diet"**. 2012. *Submitted to Microbial Ecology*

In this paper the gut microbiota was investigated in lean cloned and non-cloned pigs' that received HF/HE diet through 17-21 weeks of intervention study. The gut microbiota from colon and terminal ileum of lean and obese, cloned and non-cloned pigs was investigated by qPCR (RotorGene) and qPCR (fluidigm). The results revealed that lean cloned and non-control pigs had a different overall composition of their gut microbiota at endpoint as estimated by PCA analysis. The ratio of *Firmicutes* to *Bacteroidetes* was higher in the lean group than the obese in colon microbiota.

Main finding: HF/HE diet changes the gut microbiota in the absence of obesity.

The gut microbial composition of lean and obese cloned and non-cloned pigs on high-energy diet

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Abstract

Cloned animals are considered to be genetically identical and may provide a good model for obesity-related studies due to elimination of biological variations, resulting in a more controlled experimental animal model. The microbiota of colon and terminal ileum was evaluated in cloned and non-cloned control pigs that received a high-energy diet with either restricted or *ad libitum* access to feed, resulting in lean or obese phenotypes, respectively. The fecal microbiota of lean animals was investigated by terminal restriction fragment length polymorphism (T-RFLP), whereas the intestinal microbiota of both lean and obese clones and controls was analyzed by quantitative real time PCR and a high-throughput qPCR platform (Fluidigm) tagging multiple bacteria at different phylogenetic levels. Principal component analysis (PCA) of the T-RFLP profiles revealed that lean clones and controls had a different overall composition of their gut microbiota at endpoint. The bacterial diversity, however, was similar between lean clones and controls over time as estimated from T-RFLP profiles by the Shannon-Weaver index. The colon of lean clones contained relatively more *Firmicutes* and less *Bacteroidetes* than obese clones as estimated by qPCR. Analyses of intestinal microbiota by Fluidigm showed differences in specific bacterial groups between lean and obese animals. Our results show that the overall composition but not the diversity of the fecal microbiota differed between lean cloned and lean control pigs. The gut-microbial analysis of all the lean pigs suggests that high-fat/high-energy diet is associated with changes in the gut microbiota in the absence of obesity.

Key words

Cloned pigs, biological variation, obesity, high-fat/high-energy diet, gut microbiota

Background

Animals are frequently used as models for human obesity-related studies and therefore it is important that these animals have human-like physiology, in order to understand the underlying causes of obesity in humans. Rodents are the animals of choice for many experimental and interventional studies in relation to human diseases even though pigs have more similarities to humans especially in regard to gastrointestinal physiology. The reason for the use of rodents as animal models is because rodents are easily produced in large numbers and are cost effective while this may be difficult in case of larger animals such as pigs [1]. In nutritional and obesity related studies, rodents are frequently used as animal models especially in studying obesity related gut microbiota [2-4]. Despite this fact, efforts have been made to use pigs as animal models for studying obesity and gut microbiota [5,6]. Pigs are generally considered good animal

models for studying human diseases due to their similar physiology as humans [7] but one factor in such experimental studies is biological variations between animals. Cloning of animals such as pigs may provide an animal model with smaller inter-individual variation than normally bred siblings thereby enhancing experimental control and standardization and also possibly reducing the number of animals needed in experimental studies. Cloning of pigs has been performed previously mainly by somatic cell nuclear transfer [8-11] but this method often results in low number of cloned animals due to loss of embryos either during the gestation period or death of the piglets shortly after birth [10,12]. It has been suggested that cloning gives rise to altered metabolic characteristics [13,14], skin abnormalities [11] and other physiological defects [15], which may limit the use of cloned animals as experimental models.

Although pigs have been used to investigate the effect of obesity on gut microbiota, the numbers of studies are limited. As mentioned before, rodents are more frequently used animal models in obesity-gut microbiota studies and recently one of the ground breaking studies in this subject was performed using germ-free mice by Bäckhed and colleagues [16]. The germ-free mice had an increase in body-fat percent after being colonized with the gut microbiota of conventionally raised mice [16], although germ-free mice have been shown to be protected against diet-induced obesity [3]. These studies reveal that the gut microbiota is an important factor that affects energy storage in the body, possibly in part due to an increased energy-harvest by the microbiota. Other studies have shown that the microbiota influences the energy balance, thereby possibly playing a role in development of obesity [17]. Furthermore, obesity-related gut microbiota has also been shown to be associated with a reduction in bacterial diversity [18]. In high-fat (HF) diet-induced obesity, nutrient load presumably affects the composition of the microbiota as shown in mice, humans and pigs [2,4,5]. This in turn causes changes in the dominance of one or more bacterial division, especially reducing the number of bacteria belonging to the phyla *Bacteroidetes* and increasing the number of bacteria belonging to the phyla *Firmicutes*. Both *Bacteroidetes* and *Firmicutes* produce short chain fatty acids (SCFA) from digestion of otherwise indigestible dietary compounds which in turn provide their host with extra energy [17,19]. These studies together suggest that the microbiota of obese individuals extracts energy from the diet more efficiently than the microbiota of lean individuals. Other studies have been performed to find this putative relation between diet-induced obesity and changes in specific groups of bacteria [20,21]. Recently an association between obesity and an increase in *Lactobacillus reuteri* was found in humans [20] but the correlation between specific bacterial species and obesity remains unclear. This relation between the composition of the microbiota and obesity needs further investigation in order to increase understanding of cause and effect in the complex interaction between microbiota, body-fat, diet and age.

The aims of the current study was to investigate i) if the microbiota of cloned pigs have smaller biological variation than that of non-cloned pigs; ii) the interaction between diet, age and obesity using cloned pigs and non-cloned pigs of both lean and obese phenotypes, iii) to what extent the bacterial diversity and the initial composition of the microbiota is changed in lean cloned and non-cloned pigs fed a restricted high-energy diet and iiv) the relative abundance of different phylogenetic groups of bacteria in colon and terminal ileum in lean and obese, cloned and non-cloned pigs.

Methods

Animals

The cloning experiments were performed as described previously [13,22] using donor cells obtained from cultured ear fibroblasts from a Danish Landrace (L) x Yorkshire (Y) (65% x 35%) sow. The cloned embryos were subsequently transferred surgically to surrogate sows (recipients) five to six days after cloning [13]. Five surrogate sows gave birth to a total of 17 female cloned pigs, during a period of three years. The non-cloned pigs (n=19) (75% L x 25% Y) were obtained from 6 sows after standard artificial insemination. The pigs were reared in the experimental stables at University of Aarhus (Tjele, Denmark) and the studies were approved by the Danish Animal Experimental Committee.

Experimental set up

The cloned pigs (n=8) (clones) and non-cloned pigs (n=9) (controls) that were allocated as the lean group were delivered vaginally and the pigs were subsequently nursed by the sows for four weeks. After weaning all pigs received a standard pig diet with an energy distribution of 18.5% protein, 7.9% fat, 72.4% carbohydrate and 1.2% fibre, until the cloned pigs were 22 weeks old and the non-cloned pigs were 19 weeks old. After this period, the pigs were housed individually and fed a wheat-based high-fat/high-energy (HF/HE) experimental diet consisting of 19.5% protein, 27% fat, 53% carbohydrates and 0.5% fibre [14] in restricted amounts for 17 and 21 weeks, respectively. The lean group of pigs received the same diet as the pigs in the obese group (*ad libitum*) but restricted to 60% of the feed consumed by the *ad libitum* group.

The pigs that were allocated as the obese group, consisted of cloned pigs (n=9) (clones) and non-cloned pigs (n=10) (controls) which were given HF/HE diet with *ad libitum* access to feed. In this group, four cloned pigs and four non-cloned pigs were vaginally delivered while the remaining pigs were delivered by caesarean section. All the pigs were nursed for four weeks and subsequently received standard diet for the next nine weeks. The pigs were then housed individually and received a HE diet [14] and fed *ad libitum* for the following 20 weeks. All the pigs were euthanized after 24 hours of fasting. Cloned pigs were euthanized at 39 weeks of age and the non-cloned pigs were euthanized at 40

weeks of age. At the end of the experiment, the body-fat composition of the pigs was measured by Computed Tomography (CT) scanning.

Sampling and tissue collection

Fecal samples were collected from rectum on the day before the pigs received the experimental diet (baseline samples) and samples were then collected biweekly until the animals were euthanized. The fecal samples taken at the end of the study are mentioned as endpoint samples throughout the manuscript. Tissue samples from terminal ileum and distal colon with content (digesta) were also collected at the end of the study. The samples were immediately frozen in liquid nitrogen and stored at -80ºC for later analyses.

DNA extraction

DNA was extracted from the fecal samples taken every four week from lean cloned (n=8) and non-cloned pigs (n=9) starting at 22 weeks of age (baseline) until they were euthanized after the diet-intervention period (endpoint). The same DNA extraction method was used to extract DNA from digesta of distal colon and terminal ileum obtained at the end of the study from all cloned pigs (n=17) and non-cloned pigs (n=19).

The DNA was extracted from 200 mg fecal and digesta sample using the QIAamp DNA stool mini kit (QIAamp DNA Stool Mini Kit, Qiagen, Hilden, Germany) according to manufacturer's instructions. An additional bead beating step for 2 min was added in order to disrupt the cell wall of Gram-positive bacteria. DNA concentrations were measured by spectrophotomety (NanoDrop Technologies, Wilmington, DE, USA).

The analysis of fecal microbiota by T-RFLP

The microbiota in fecal samples from cloned pigs (n=8) and non-cloned pigs (n=9) in the lean group was characterized by terminal restriction fragment length polymorphism (T-RFLP) as described previously [23]. The extracted DNA samples were diluted in nuclease-free water to obtain a final concentration of 5 ng μl^{-1} and a PCR was performed in duplicates as described previously [23]. The primers that targeted most bacterial 16S rRNA gene were, Eub-8fm (5'-AGAGTTTGATCMTGGCTCAG- 3') labeled with 5´FAM and Eub-926r (5-'CCGTCAATTCCTTTRAGTTT- 3') (DNA Technology, Aarhus, Denmark) [23]. PCR mix of a total of 50 µl per sample consisted of 5 µl Fermentas Taq-buffer, 4 µl MgCl2 (25 mM), 2.0 µl (10 µM) deoxyribonucleotide triphosphate (dNTP), 0.5 µl Fermentas Taq polymerase (2.5 Unit µl-1), 0.5 µl of each primer (20 µM), 35.5 µl nuclease-free water and 2 µl DNA sample. The cycling conditions were: an initial denaturing step at 940C for 6 min followed by 32 denaturing cycles at 940 C for 45 s, annealing at 560 C for 45 s, an extension step at 720 C for 2 min, and a final extension at 720 C for 10 min. The PCR products were then verified by gel

electrophoresis adding 10 µl sample pr. well. The PCR products were purified by High Pure PCR purification kit (Roche Applied Sciences, Mannheim, Germany) and 200 ng of this PCR product was digested with 2.0 µl of the restriction enzyme *HhaI* (Promega Corporation, Madison, USA) at 37°C for 3 h. A mixture of the digested PCR products (2 µl), formamide (10 µl) and 0.50 µl Megabase ET900-R size standard (GE Health Care, Buckinghamshire, UK) was subsequently run in duplicates on a capillary electrophoresis genetic analyser (Applied Biosystems Genetic Analyzer 3130/3130xl, Foster City, CA, USA).

The terminal restriction fragments (T-RFs) were analyzed using the BioNumerics software (Applied Maths, Kortrijk, Belgium). The T-RF profiles were aligned against an internal standard. T-RF fragments with difference less than two base pairs were considered identical and therefore excluded. The bands present in both duplicates were accepted as bacterial fragments and the duplicate with the best intensity was chosen for microbial profiling. T-RF profiles were subsequently identified *in silico* using the MiCA on-line software [24] and 16S rRNA gene sequences were assigned to their taxonomic names on the Ribosomal Database project [25]. All the data was transferred to Microsoft Excel for further analysis. In Microsoft Excel intensities less than 50 were removed and the relative intensity of a given T-RF, in the range of 60-800 bp, was calculated by relating the sum of the intensity of a given T-RF in the entire group of animals to that of the total intensity of all T-RFs in the samples. For both graphical presentations and principal component analysis (PCA), only the predominant T-RFs with a mean relative intensity above 1 percent were considered.

Statistical analysis of T-RF profiles

The T-RFs between 60 and 800 bp were imported into the statistical software programs Stata 11.0 (StataCorp, College Station, TX) and Unscrambler version 9.8 (CAMO, Oslo, Norway). Comparisons of group differences in the overall microbial communities between cloned and non-cloned pigs at the different sampling points were investigated with PCA. In these models, the T-RFs were standardized (centred and 1/SD) prior to the modelling phase to ensure that all the T-RFs equally influence the models, and possible outliers were inspected visually and with *Hotelling* T^2. The Shannon-Weaver index of diversity (H') was used to estimate the diversity of the bacterial fragments as described previously [26] and was calculated based on all of the initial T-RFs. At each of the sampling points, comparisons of cloned and non-cloned pigs with respect to this index were evaluated using the Mann Whitney U-test. Group differences were considered statistically significant when $P < 0.05$.

Quantification of *Bacteroidetes* and *Firmicutes*

Quantitative real time PCR (qPCR) was performed on 16S rRNA gene extracted from terminal ileum and distal colon content for quantification of the relative abundance of the phyla *Bacteroidetes* and *Firmicutes*. The PCR conditions such as annealing temperature and MgCl2 were optimized. Pure cultures of three bacteria; *Clostridium perfringens* (NCTC 8449), *Odoribacter splanchnicus* (isolate DJF_B089) and *Escherichia coli* (ATCC 25922) were used to establish standard curves for each primer set. Ten fold serial dilution of all DNA samples were made from 2.5 x102 ng µL-1 to 2.5x10-6 ng µL-1. Furthermore, serial dilutions of 16S rRNA gene corresponding to the above dilutions from two random samples obtained from the colon and terminal ileum content of cloned and non-cloned pigs to construct standard curves, to further verify if PCR inhibitors were present in extracted DNA.

qPCR primers and conditions

For qPCR the phyla specific primers by Bacchetti De Gregoris et al. (2011) [27] were used. The annealing temperature and MgCl2 were optimized for the thermocycler used in this study (Rotor-Gene Q Real Time PCR cycler (Qiagene)). A universal primer set with an amplicon length of 147 bp (S-D-Bact-0907-a-S-20 5'-AAACTCAAAGGAATTGACGG-3'; S-D-Bact-1054-a-A-20 5-' ACGAGCTGACGACAGCCATG-3') [28] was used to detect all bacteria. The specific primer sets were: *Bacteroidetes* primer set with an amplicon length of 240 bp (798cfbF 5' CRAACAGGATTAGATACCCT'3 and cfb967R 5' GGTAAGGTTCCTCGCGTAT '3) and *Firmicutes* primer set with an amplicon length of 200 bp (928F-Firm 5' TGAAACTYAAAGGAATTGACG '3; 1040firmR, 5' ACCATGCACCACCTGTC '3) [27]. The qPCR reactions contained 12.5 µl of SYBR® Green JumpStart™ Taq ReadyMix™ without MgCl2 (Sigma-Aldrich, MO, USA), 0.3 µmol l-1 of each primer and 5 µl template DNA adjusted to 5 ng µL-1. MgCl2 concentration was optimized and a final concentration of 2.5 mM MgCl2 per reaction was chosen. All PCR conditions were subsequently verified by conventional PCR and gel electrophoresis. A non template control (NTC) was included in all runs to detect contamination and DNA artifacts. At the end of each run a melting curve analysis was performed for each individual sample to detect presence of primer-dimers.
qPCR was performed with an initial denaturing step of 10 min at 95°C, 95°C for 30 s, 35 cycles of 56°C for 20 s and an elongation step of 72°C for 20 s. The threshold cycle (Ct) and the bacterial enumeration (DNA copies µl-1) were determined automatically by Rotor Gene software (Rotor-Gene Q 2.0.2 (Qiagene)).

A standard curve was constructed for each primer set from serial dilution of extracted DNA from specific bacteria: *C. perfringens, O. splanchnicus* and *E. coli*. The relative abundance of the 16S rRNA gene from bacteria belonging to the phylum *Bacteroidetes* and the phylum *Firmicutes* was calculated against the 16S rRNA gene obtained from all bacteria

and the relative abundance of bacteria from the two phyla in each pig was subsequently calculated. Group differences were investigated with Mann Whitney U-test and the correlation analyses were performed by Spearman's rank-order correlation in GraphPad prism version 5.00 for Windows (GraphPad software, San Diego CA USA). A statistical significance was considered when $P < 0.05$.

Ileal and colonic microbial identification by 48.48 dynamic array

High throughput quantitative real time PCR (qPCR) was performed using a 48.48 Dynamic Array Integrated Fluidic Circuits (Fluidigm, CA, USA) which combines 24 primer sets with 48 samples to run 2304 simultaneous qPCR reactions. This Fluidigm chip was designed by (Hermann-Bank et al. 2012 (in prep)) in which the primer sets target 16S rRNA gene of different bacterial phylogenetic groups (Domain, Phyla, Class, Family, Genus and Species level, respectively) and normalizes each group according to a universal primer set. The samples consisted of 20 µM forward and reverse primers, 1x Assay loading reagent (Fluidigm, PN85000746), 1x low EDTA TE buffer (VWR, APLIA8569.0500) and master mix consisting of: 20 x DNA binding dye sample loading reagent (Fluidigm, PN 100-0388), 20 x EvaGreen DNA binding dye (Biotium, PN 31000) and 2 x Taqman master mix (Applied Biosystems). The concentrations of 16S rRNA gene were optimized and subsequently samples were adjusted to a concentration of 50 ng µl-1. In each chip a non template control (NTC) was included to detect any contamination or non-specific amplification. For details on qPCR reaction mix and cycling conditions as well as all 24 primer sets we refer to Hermann-Bank et al. (in prep). The obtained Ct values were subsequently exported to Microsoft Excel for further analysis. The relative proportion of bacteria representing each taxon was calculated based on the Livak method [29]. Hence, the relative quantifications of the PCR signal of the target 16S rRNA gene in the obese pigs was related to that of the lean pigs which were considered to harbor the reference composition of the gut microbiota. Similarly, in comparisons between cloned and non-cloned pigs, the latter were considered to be the reference. Fold differences in the different bacterial groups are subsequently calculated by $2^{-\Delta\Delta Ct}$ and log2-transformed for visual appraisal in the figures. All the statistical analysis and figure arts were performed on GraphPad prism version 5.00 for Windows (GraphPad software, San Diego CA USA).

Results

Weight and body-fat lean pigs

The cloned pigs of lean phenotype weighed 65.1 kg (± 7.4) at beginning of the diet-intervention period (baseline; age: 22 weeks) and non-cloned pigs (age: 19 weeks) weighed 61.7 kg (±1.4). The pigs were fed a restricted HF/HE diet and

were given 60% of the feed consumed by pigs fed *ad libitum* throughout the diet-intervention period. At the end of the experiment, the cloned pigs (n=8) and non-cloned pigs (n=9) weighed 127.1 kg (±5.9) and 119.1 kg (±3.2), respectively. In the obese group, all pigs were fed a HE diet *ad libitum* throughout the diet-intervention period. At the beginning of the experiment (baseline), the cloned pigs (age: 13 weeks) had an average weight of 38 kg (± 4.1) and the non-cloned pigs weighed 38 kg (± 2.3). By the end of the diet-intervention experiment, the obese cloned pigs (n=9) had an average weight of 147.5 kg (±5.9) and obese non-cloned pigs (n=10) weighed 170.1 kg (± 5.4). The weight of lean non-cloned pigs was significantly lower than that of the obese non-cloned pigs ($p<0.0001$) and the same was observed for cloned pigs ($P<0.02$). CT scans of the lean and obese pigs showed that the obese non-cloned pigs had a higher percentage of body-fat than the lean non-cloned pigs ($P<0.0004$) and the same was observed in the obese cloned pigs ($P<0.03$) (Fig.1).

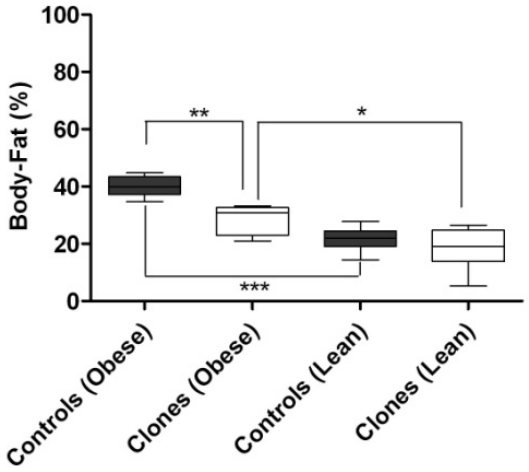

Fig. 1 Percent body-fat in lean and obese, cloned and non-cloned pigs (statistics performed by Mann-Whitney U test, * indicate significance for $p<0.05$)

The fecal microbiota of lean cloned pigs and non-cloned pigs

T-RFLP was used to profile the composition of the fecal microbiota and PCA of the most predominant terminal restriction fragments (T-RFs) (> 1%) revealed that the lean cloned pigs and non cloned pigs had a different overall composition of their fecal microbiota from the start of the experiment (baseline) until the pigs were euthanized at the age of 39 and 40 weeks (endpoint), respectively (Fig. 2). The bacterial diversity of the fecal microbiota was similar between lean cloned and non-cloned pigs at both sampling times, as estimated by the Shannon-Weaver index.

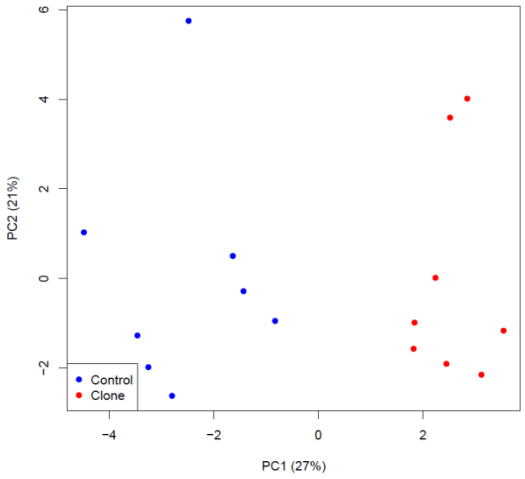

Fig. 2 Principal Component Analysis (PCA) of the most predominant T-RFs in cloned (clones) (●) and non-cloned pigs (controls) (○) at endpoint

Furthermore, there was no change in the diversity of the fecal microbiota determined every four weeks throughout the diet-intervention period in both lean cloned and non-cloned pigs. A comparison between the microbiota of the lean cloned and non-cloned pigs according to all the T-RFs (bacterial load) did not show any significant difference at baseline ($P<0.7$) (Fig. 3a). At endpoint one particular T-RF (584 bp) was significantly higher than all the other T-RFs in the non-cloned pigs ($P<0.03$) (Fig. 3b) which may represent bacteria belonging to the phylum *Firmicutes*. Based on relative abundance of all the T-RF base pairs, i.e. total bacterial abundance, no difference was observed in lean cloned pigs at sampling time before the start of diet intervention or at the end of diet-intervention and the same was observed in lean non-cloned pigs.

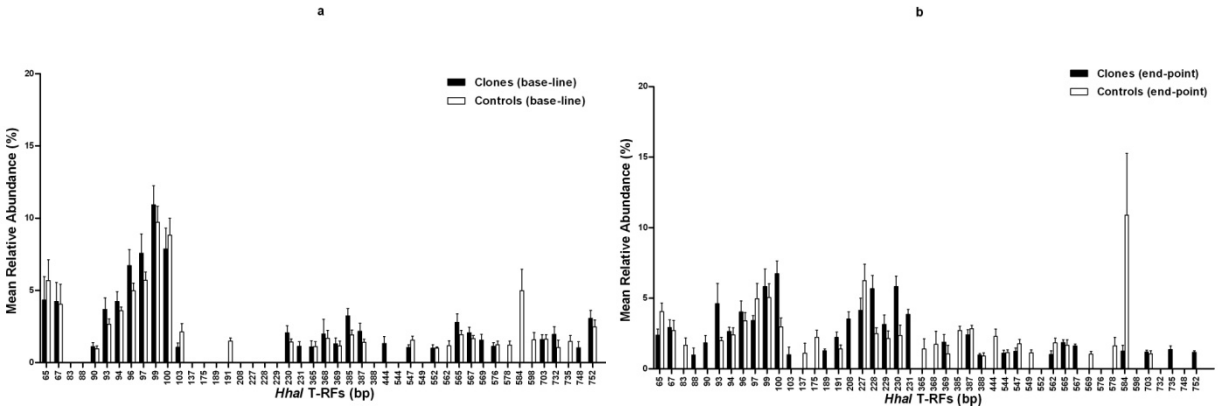

Fig. 3 Mean relative abundance of the most predominant T-RFs (>1%) in the fecal samples at baseline (a) and endpoint (b) in cloned (clones) (■) and non-cloned pigs (controls) (□). The error bars represent standard deviation.

Several T-RFs' with mean relative abundance of more than one percent were present in lean cloned pigs at baseline but absent at endpoint. There was a clear distinction between the individual T-RFs at baseline (Fig. 3a) and endpoint in both lean cloned pigs and lean non-cloned (Fig. 3b). The relative abundance (>1%) of T-RFs 93 bp to 100 bp at baseline was

higher than the relative abundance at endpoint in both lean cloned pigs and lean non-cloned pigs and the subsequent in silico analysis of these T-RFs indicated that these T-RFs probably represent bacteria belonging to the phylum *Bacteroidetes* (Fig. 3a).

In fecal samples from lean cloned pigs the 28 most predominant T-RFs (>1%) out of a total of 79 T-RFs represented 82% of all the original bacterial fragments at baseline (Fig. 3a). In lean non-cloned pigs at baseline, the 30 most predominant T-RFs out of 90 original bacterial fragments represented 81% of the total bacteria (Fig. 3a). At endpoint fecal samples from lean cloned pigs, 31 most predominant T-RFs constituted 83% of all 84 bacterial fragments and in lean non-cloned pigs 32 out of 74 bacterial fragments represented 80% of all bacteria in fecal samples (Fig. 3b). These results show that there were no differences in overall bacterial load between baseline or endpoint fecal samples in both lean cloned and non-cloned pigs.

Relative abundance of *Firmicutes* and *Bacteroidetes* varies between lean and obese pigs

The relative abundance of *Firmicutes* and *Bacteroidetes* in microbiota from colon and terminal ileum was not different between lean cloned and non-cloned pigs. The same was observed between obese cloned and obese non-cloned pigs. The microbiota in colon of lean vs. obese cloned pigs revealed that the lean cloned pigs had a higher abundance of *Firmicutes* (P< 0.01) (Fig.4a) and a lower abundance of *Bacteroidetes* (P<0.0007) than the obese cloned pigs (Fig. 4b). In the non-cloned group of pigs, the relative abundance of *Firmicutes* in colon was higher in lean non-cloned pigs than in obese non-cloned pigs (P<0.02) (Fig. 4a) while no difference was observed in relative abundance of the phyla *Bacteroidetes* between lean and obese non-cloned pigs (Fig. 4b).

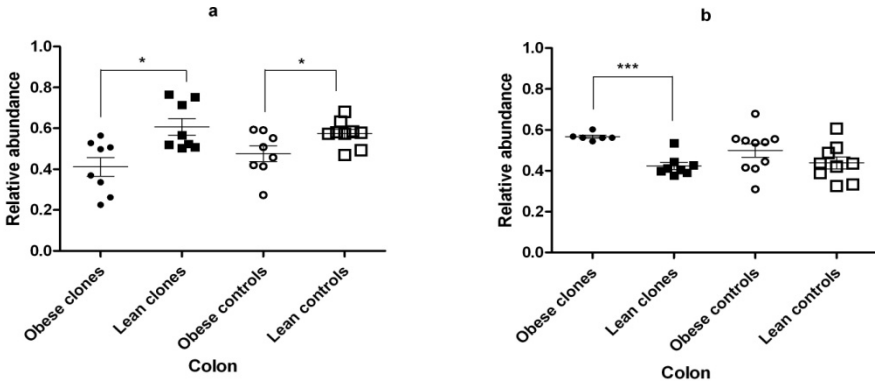

Fig. 4 Comparison of relative abundance of *Firmicutes* (a) and *Bacteroidetes* (b) in colon of lean and obese, cloned (clones) and non-cloned (controls) pigs. Obese clones (●), lean clones (■), obese controls (○), and lean controls (□). Statistics performed by Mann-Whitney U test and significant differences are indicated by * for p<0.05.

The relative abundance of *Bacteroidetes* in colon was significantly higher than terminal ileum in both lean cloned pigs (P<0.0002) and obese cloned pigs (P<0.0004) (Fig. 5a). The same was observed in both lean non-cloned (p<0.0001) and obese non-cloned pigs (P<0.0003) (Fig. 5) (Fig. 5b). In the lean cloned pigs (Fig. 5c) as well as lean non-cloned pigs (Fig. 5d), there was no difference in the abundance of *Firmicutes* in either colon content or ileum content (Fig. 5). In the obese cloned and obese non-cloned pigs, a higher abundance of *Firmicutes* was observed in terminal ileum (P<0.03, P<0.02 respectively) than in colon (Fig. 5c-d).

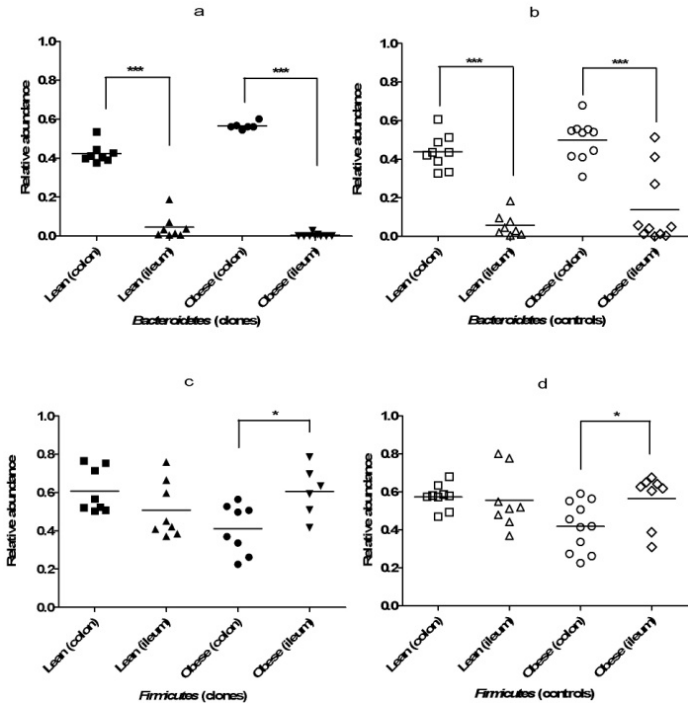

Fig. 5 Comparison of relative abundance of *Firmicutes* and *Bacteroidetes* between colon and terminal ileum of lean and obese cloned (clones) and non-cloned pigs (controls). Lean clones (colon) (■), lean clones (ileum) (▲), obese clones (colon) (●), obese clones (ileum) (▼), lean controls (colon) (□), lean controls (ileum) (Δ), obese controls (colon) (○) and obese controls (ileum) (◊). Statistics performed by Mann-Whitney U test and significant differences are indicated by * for p<0.05.

The microbiota in terminal ileum and colon of lean and obese pigs

In the obese cloned pigs (colon) the relative abundance of *Lactobacillaceae* and *Cl. cluters IV* were respectively 2.6 and 2.3 fold higher as compared to lean cloned pigs, however only *Cl. cluters IV* was significantly different (P<0.03) (Fig. 6a). Overall the colonic microbiota of the obese cloned pigs had a lower abundance of ε-Proteobacterea, Streptococcaceae, *Enterobacteriaceae* (P<0.04), *Bacteroides* (P<0.03), *Cl. perfringens* and *E.coli* compared to lean cloned pigs (Fig. 6a). In terminal ileum of obese cloned pigs the relative abundances of *Streptococcaceae*, *Enterobacteriaceae* and *E.coli* were 6.3, 1.3 and 1.4 fold higher than the lean cloned pigs, respectively (Fig. 6b). In the obese non-cloned pigs the relative abundance of *Lactobacillaceae* in colon was almost 12.9 fold higher than the lean non-cloned pigs (p=0.001) (Fig. 6c). While *Streptococcaceae* (p=0.003) and *Bacteroides* (p=0.0007) were significantly lower in colon of obese non-cloned

pigs as compared to the lean non-cloned pigs (Fig. 6c). In terminal ileum of obese non-cloned pigs, the relative abundance of *ε-Proteobacterea*, *Enterobacteriaceae* and *Cl.cluster XIV* were 1.3 fold, 1.5 fold and 2 fold higher than the lean non-cloned pigs (Fig. 6d), however these were not significant.

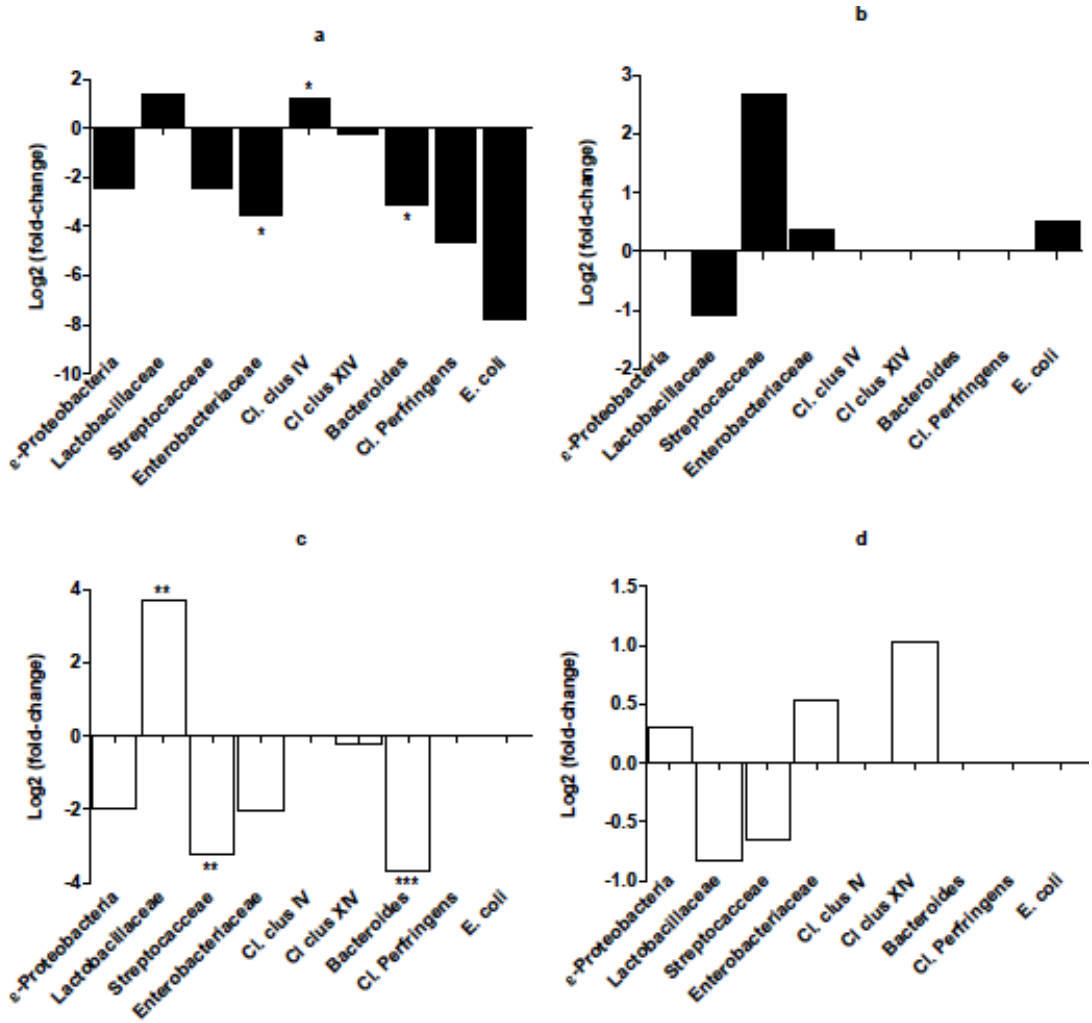

Fig. 6 Comparison of obese and lean pigs in each group by the ratio (fold-differences) of the 16S rRNA gene of different bacteria groups in microbiota of obese cloned pigs' colon (a), ileum (b) and obese control pigs' colon (c) and ileum (d). The fold-changes are compared against the lean pigs for each group. Statistics performed by Mann-Whitney U test and significant differences are indicated by * for $p<0.05$.

Comparing the microbiota of cloned and non-cloned pigs, in the colon of obese cloned pigs, the relative abundance of *Lactobacillacea*e, *Cl. Cluster IV, Cl. Cluster XI*V and *Cl. perfringens* were 1.5, 1.4, 1.2 and 3.1 fold higher than the colon of obese non-cloned pigs (Fig. 7a), however these were not significant. In terminal ileum of obese cloned pigs, contained 11 ($P<0.04$) fold higher relative abundance of *Streptococcaceae* as compared to non-cloned pigs (Fig. 7b).

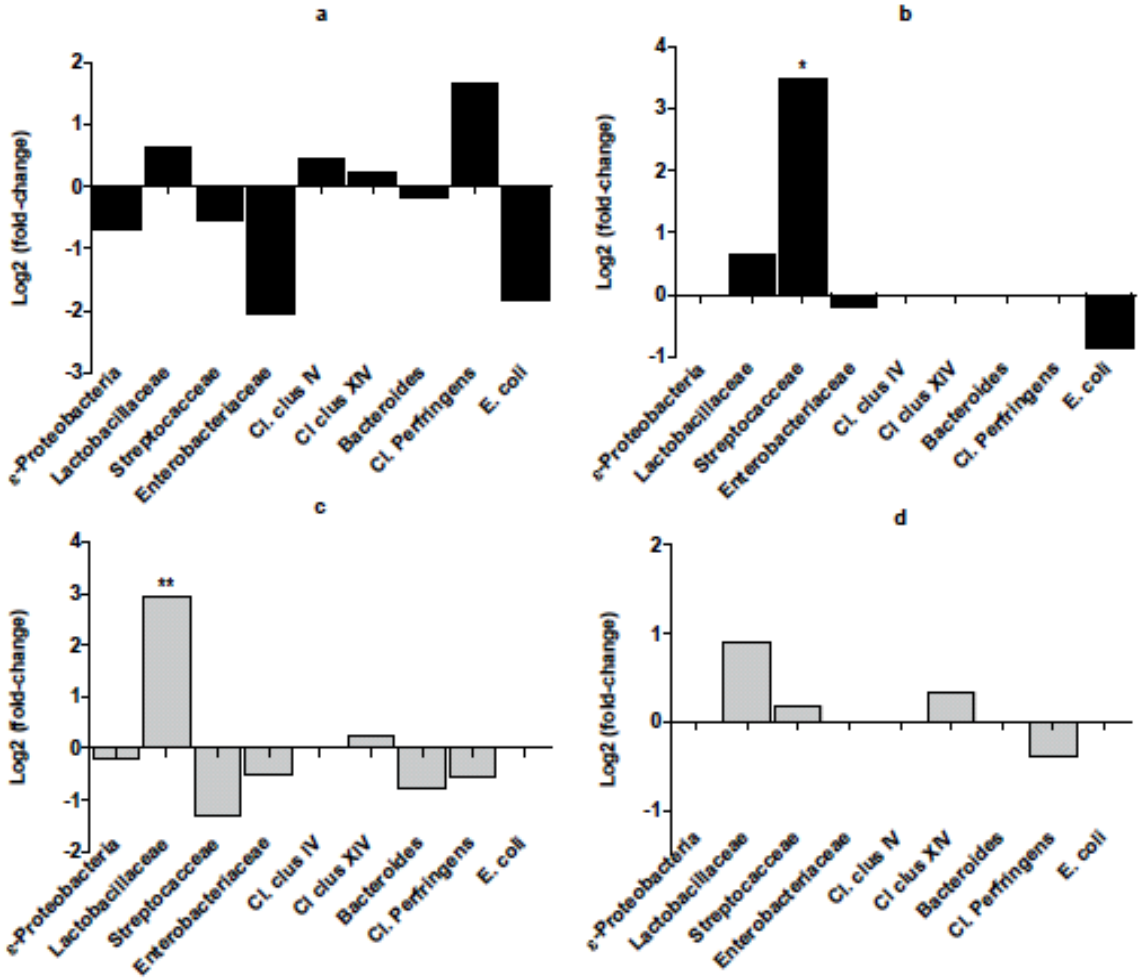

Fig. 7 Comparison of cloned and non-cloned pigs' microbiota of selected bacteria by calculating the ratio (fold-differences) of the 16S rRNA gene of different bacteria groups (colon (a) and ileum (b) of obese clones vs. obese and colon (c) and ileum (d) lean clones vs. lean controls. The fold-changes are compared against the non-cloned pigs. Statistics performed by Mann-Whitney U test and significant differences are indicated by * for $p<0.05$.

When comparing the lean cloned and non-cloned pigs, in colon of cloned pigs there was a 3 fold higher relative abundance of *Lactobacillaceae* ($P<0.008$) than in colon of control pigs (Fig. 7c). In terminal ileum of lean cloned pigs, the relative abundance of *Lactobacillaceae* was 2 fold higher; however this was not significant (Fig. 7d).

Discussion

In this study an experimental animal model employing cloned pigs was investigated in order to reduce biological variation between the pigs and to further elucidate the relation between HF/HE diet and the composition of the microbiota in fecal samples of lean pigs. We also investigated the microbiota of colon and terminal-ileum in lean and obese pigs. The same pigs have also been used by other groups in metabolomic [13,14] and innate immunological studies [30] to obtain a better understanding of the underlying causes of obesity. In our study we found that the lean cloned pigs on restricted diet had a different overall fecal microbial composition than the lean non-cloned pigs both at

the start of the diet-intervention study i.e. baseline and in particular at the end of the diet intervention period i.e. endpoint. Based on the microbial profiles of the lean cloned and non-cloned pigs we did not find any evidence of smaller biological variation among the cloned pigs as compared to non-cloned pigs which was also found previously by other groups [13,14]. There could be many reasons for the variations observed among the cloned pigs. One explanation could be that in the process of cloning, a single somatic cell nucleus is introduced to an enucleated oocyte which contains the maternal mitochondria and therefore maternal mitochondrial effect could cause the inter-individual variation between the cloned pigs. Other studies on cloned pigs have also pointed out variations in different phenotypic characters such as skin abnormalities [11] and other physiological defects [15] among the cloned pigs and compared these phenotypic characters to the non-cloned control pigs [31].

Characterization of the gut microbial community revealed that the overall levels of bacterial diversity did not change from beginning of the diet intervention period to end of the intervention study in both lean cloned pigs and lean non-cloned pigs. This has been shown in a previous study where the overall bacterial diversity did not change over time while the individual bacterial divisions may change [32]. The phyla *Firmicutes* and *Bacteroidetes* were the two most abundant groups of bacteria in the microbiota of pigs [33]. By comparing the lean and obese pigs in our study, it was found that the lean cloned pigs had a higher abundance of the phyla *Firmicutes* and correspondingly less abundance of bacteria belonging to the phyla *Bacteroidetes*. The same observations were made in the lean non-cloned pigs with higher abundance of *Firmicutes* and lower abundance of the phyla *Bacteroidetes* in the lean non-cloned pigs as compared to obese non-cloned pigs. These findings are in contrast to results obtained in mice [2,34], pigs [5] and humans[4], where the lean individuals had higher abundance of *Bacteroidetes* as compared to their obese counterparts. As the lean animals in our study received HF/HE diet in restricted amounts, this diet could have affected the gut microbial community even in the absence of obesity and could explain the higher abundance of *Firmicutes* and lower abundance of *Bacteroidetes* found in the lean group. This suggests that ingestion of high-energy diet can affect the composition of the microbiota independent of weight or body-fat composition. Alterations in the microbial community upon switch to a high-energy diet in absence of obesity have been shown before [35] and are in agreement with these findings. Interestingly, the obese cloned pigs had a lower abundance of *Firmicutes* in colonic microbiota compared to lean cloned pigs despite their higher body weight and body-fat percent compared to the lean group. One explanation for these results could be alterations of metabolic parameters observed in obese cloned pigs. Clausen et al. [13] and Christensen et al. [14] found high levels of bile acid in these obese cloned pigs which could be due to alteration in mechanisms that regulates bile acid transportation due to cloning. Zhang et al. [36] have recently shown a

connection between alterations in organic anion transporting poly peptides (Oatps) that are responsible for transport of compounds such as bile and changes in the composition of gut microbiota in mice. They suggested that dysfunction of liver and bile transporting mechanisms causes overgrowth of bacteria belonging to the phyla *Bacteroidetes* and a concurrent reduction of bacterial abundance in the *Firmicutes* phyla [36]. In our study the higher proportion of *Bacteroidetes* observed in obese cloned pigs could be due to alterations or dysfunction of Oatps that could have affected bile acid transport and thereby caused the changes observed in the obese cloned pigs' microbial community. The metabolic phenotypes of these cloned pigs have been shown to be different from the non-cloned pigs [13,14] and these differences in metabolites could have affected the gut microbiota. As opposed to the cloned pigs, the relative abundance of *Bacteroidetes* in non-cloned pigs was not different between the lean and obese non-cloned pigs which further support that the cloned and non-cloned pigs have different phenotypes.

There are conflicting reports on the obesity related changes in abundance of *Bacteroidetes*. In some studies using lean and obese mice a positive correlation between the relative abundance of *Bacteroidetes* and increase in weight [19,37] has been reported while other studies have shown a reduction in the number of *Bacteroidetes* in obese mice compared to lean mice [2,38]. Many factors may explain the conflicting reports on the relative proportions of *Firmicutes* and *Bacteroidetes* between different studies. One other factor is the difference in test subject or animal models used in each study and/or different methodological approaches used in different studies to quantify bacterial proportions. In this study the composition of the microbiota in lean cloned pigs has a microbial profile that resembles that of the obese animals in regard to high relative abundance of *Firmicutes* and lower relative proportions of *Bacteroidetes* perhaps due to the HF/HE diet.

We further investigated the bacterial community in different phylogenetic groups in colon and terminal ileum of lean and obese, cloned and non-cloned pigs. We found a notable increase in representation of *Bacteroidetes* in colon as compared to terminal ileum and observed the same tendency in the obese cloned and obese non-cloned pigs. These results suggest that terminal ileum has generally a lower abundance of *Bacteroidetes* than colon. The abundance of Frimicutes was higher in terminal ileum than colon in the obese pigs while this difference was not observed in the lean pigs, suggesting that HF/HE diet and obesity may result in shifts in the bacterial population along the intestinal tract.

The analysis of other bacterial groups revealed community differences between obese and lean pigs. The most noteworthy finding was the higher abundance of the *Lactobacillaceae* in obese non-cloned pigs with a 12.9 fold higher

relative abundance compared to lean non-cloned pigs. There have been reports on high abundance of *Lactobacillaceae* in fecal microbiota of obese humans [20,39]. The high level of *Lactobacillaceae* found in the microbiota in colon of obese non-cloned pigs is an important finding in regard to designing probiotics, as many probiotics strains belong to the family *Lactobacillaceae*. The relative abundances of *ε-Proteobacteria* and *Enterobacteriaceae* were higher in terminal ileum of obese non-cloned pigs and the latter was also high in cloned pigs as compared to the lean cloned pigs. Lipopolysaccharide of Gram-negative bacteria is implicated to be one of the factors resulting in a low grade inflammation observed in obese mice, called metabolic endotoxemia [40].The hypothesis is that the diet modulates the gut microbial community resulting in activation of the inflammatory cascade by the gut bacteria causing metabolic endotoxemia [41,42]. The fold increase in the mentioned Gram-negative group of bacteria observed in obese pigs is an interesting finding and needs further investigation.

As mentioned before, the alteration of metabolic parameters in cloned pigs perhaps due to cloning can explain why obese or lean cloned pigs did not have the same fold increase in the abundance of Gram-negative bacteria as observed in obese non-cloned pigs. Taken together our results suggest that HF/HE diet results in changes in the gut microbial community in several different phylogenetic groups of bacteria. Although many studies have revealed great insights into the obesity related gut microbiota, profiling of the obese and lean gut microbiota in different bacterial phylogenetic groups may provide information that can be used to develop probiotic strains which may alter the gut microbial community in obese subjects and perhaps may reverse the metabolic disorders observed in obese subjects.

Conclusion

Here we conclude that the overall composition but not the diversity of the fecal microbiota differed between lean cloned pigs and lean non-cloned pigs. Based on gut microbial profiles of cloned pigs we did not observe smaller biological variation among the lean cloned pigs as compared to non-cloned pigs. Together our results suggest that cloned pigs do not provide a better model for diet-intervention studies than conventional non-cloned pigs, due to similar levels of biological variations and higher cost as compared to conventional pigs. Gut-microbial analyses of the lean pigs, however, support the hypothesis that HF/HE diet is associated with changes in the gut microbiota in the absence of obesity. Finally, diet induced obesity caused changes in different bacterial phyogenetic groups, specially an increase in Lactobacillacea and several Gram-negative groups of bacteria in the non-cloned pigs.

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Authors' contributions

RP carried out the experimental work, data and statistical analysis and wrote the manuscript. A.D.A performed the statistical analysis on the T-RFLP (Shannon-Weaver and PCA), JS designed and conducted the diet-intervention experiments, MB contributed in designing of the experiments. All authors contributed in editing the manuscript and approved the final manuscript. All the authors declare no competing interests.

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Paper III

Pedersen R., Ingerslev HC., Cirera S., Sturek M., Alloosh M., Christoffersen BØ., Moesgaard SG., Larsen N., Boye M. **"Characterization of gut microbiota in Ossabaw minipigs and Göttingen minipigs as models of obesity and metabolic syndrome".** 2012. *Submitted to PloSOne*

In this paper the intestinal microbiota was characterised by sequencing (Illumina), qPCR (Rotorgene) and high-throughput qPCR (Fluidigm), in Göttingen and Ossabaw minipigs that are animal models of obesity and metabolic syndrome, respectively.

The sequence analysis revealed several differences in different taxonomic levels between lean and obese Göttingen and Ossabaw minipigs. High abundances of *Firmicutes* and lower *Bacteroidetes* was observed in obese Ossabaw minipigs' microbiota as compared to lean Ossabaws. In Göttingen minipigs, the lean group had higher abundance of *Firmicutes* with no difference between lean and obese in relative abundance of *Bacteroidetes* in cecum or colon.

Main finding: Diet-induced obesity caused changes in the gut microbiota of Göttingen and Ossabaw minipigs; however there were differences between the changes induced by HF/HE diet and normal chow *ad libitum* diet.

Short title: Gut microbiota in pig models of Metabolic Syndrome

Characterisation of gut microbiota in Ossabaw minipigs and Göttingen minipigs as models of metabolic syndrome

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Abstract

Background: Recent evidence suggests that the gut microbiota is an important contributing factor to obesity and obesity related metabolic disorders, known as the metabolic syndrome. The aim of this study was to assess and characterise the intestinal microbiota in two pig models of obesity namely Göttingen minipigs and the Ossabaw minipigs.

Methods and Findings: : The cecal, ileal and colonic microbiota from lean and obese Osabaw and Göttingen minipigs were investigated by Illumina-based sequencing and by high throughput qPCR, targeting the 16S rRNA gene in different phylogenetic groups of bacteria. The weight gain through the study was significant in obese Göttingen and Ossabaw minipigs. The lean Göttingen minipigs' cecal microbiota contained significantly higher abundance of Firmicutes ($P < 0.006$), *Akkermensia* ($P < 0.01$) and *Methanovibribacter* ($P < 0.01$) than obese Göttingen minipigs. The obese Göttingen cecum had higher abundances of the phyla Spirochaetes ($P < 0.03$), Tenericutes ($P < 0.004$), Verrucomicrobia ($P < 0.005$) and the genus *Bacteroides* ($P < 0.001$) compared to lean minipigs. The relative proportion of *Clostridium cluster XIV* was 7.6-fold higher in caecal microbiota of obese Göttingen minipigs as compared to lean. Obese Ossabaw minipigs had a higher abundance of Firmicutes in terminal ileum and lower abundance of Bacteroidetes in colon than lean Ossabaw minipigs ($P < 0.01$). Obese Ossabaws had significantly lower abundances of the genera *Prevotella* and *Lactobacillus* and higher abundance of *Clostridium* in their colon than the lean Ossabaws. Overall, the Göttingen and Ossabaw minipigs displayed different microbial communities in response to diet-induced obesity in the different sections of their intestine.

Conclusion: Obesity-related changes in the composition of the gut microbiota were found in lean versus obese Göttingen and Ossabaw minipigs. In both pig models diet seems to be the defining factor that shapes the gut microbiota as observed by changes in different bacteria at different phylogenetic levels.

Introduction

Obesity in humans has reached epidemic proportions worldwide, which is mainly due to a combination of inactive lifestyle and increased energy intake [1]. Obesity is a condition characterised by accumulation of fat in adipose tissue and a state of metabolic imbalance. The morbid conditions related to obesity such as abdominal obesity, insulin resistance and glucose intolerance, hypertension and dyslipidemia are together called the metabolic syndrome (MetS) [2]. Obesity and its co-associated morbidities namely cardiovascular disease, type-2 diabetes (T2D), fatty liver disease and hypertension are a great economic burden in affected countries. Recently, the gut microbiota has been implicated as one of the influencing factors that further promotes obesity and metabolic disorders in obese subjects [3,4]. The gut microbiota is affected by dietary changes and is considered to be an environmental factor affecting the energy balance and whole body

metabolism which may contribute to obesity and its metabolic disorders [3,5,6]. Recent studies have shown an altered gut microbiota characterised by reduced diversity in diet induced obese and diabetic mice as well as diabetic humans [6–10]. Other studies in mice have demonstrated changes in the composition of the gut microbiota independent of the obese state and in response to high-fat/high-energy diet, suggesting other mechanisms that may contribute to obesity and metabolic syndrome [11,12]. In addition, Cani and colleagues [13–15] have provided evidence of a causal role of gut microbiota in the low grade inflammation in the gut of mice. The gut microbiota may lead to low grade inflammation induced by the lipopolysaccharides (LPS) present on the outer membrane of Gram-negative bacteria, causing activation of the innate immune response [16]. This low grade inflammation is connected to low, but constant levels of LPS in the circulation and to increased levels of adiposity and insulin resistance [13]. Together these findings suggest that high-fat diet (HFD) and obesity are associated with gut microbiota dysbiosis leading to alterations of gut barrier and resulting in an increased level of circulating LPS and a low grade inflammation (reviewed in [17]). However most of these studies are performed in mice that are genetically obese (ob/ob mice) and due to the many differences between humans and mice these findings may not be translated directly to humans [18]. Therefore, other animal models may provide better models for obesity-gut-microbiota related studies. Pigs are generally considered to be excellent biomedical models for nutritional studies and their gastrointestinal tract and physiology have similarities to humans, making pig an excellent animal model in obesity related studies [18]. Pigs have previously been used in a few obesity studies to investigate their gut microbiota [19,20]. Minipigs have a potential of becoming excellent animal models for gut microbiota related studies, due to their smaller size than domestic pigs. In this study we investigate the composition of the gut microbiota in relation to obesity and MetS in two pig models, Göttingen and Ossabaw minipigs. Female Ossabaw minipigs when fed high-fat/cholesterol, high-caloric diet develop symptoms of MetS with abdominal adiposity, glucose intolerance, insulin resistance and cardiovascular disease (CVD) [21–23] and therefore Ossabaw minipigs are considered one of the relevant animal models for obesity and MetS [24]. Göttingen minipigs are also used in diabetes research and are proposed to be good animal models for studying obesity and metabolic disorders caused by obesity [25–27]. To our knowledge the gut microbiota of Ossabaw minipigs have not been characterised previously either in lean or obese states. Therefore, the objective of this study was to investigate the effect of obesity/high-energy diet on the composition of the gut microbiota in the two pig models of obesity. In Ossabaw minipigs the dietary regimen was very different between lean and obese animals and any differences found between these two groups will be a combined effect of obesity and different diet. In the Göttingen minipigs the diets did not differ much between lean and obese minipigs, and group differences will most likely primarily be due to differences in obesity. We characterised the gut microbiota of Ossabaw and Göttingen minipigs by Illumina-based sequencing and further confirmed our findings using a high-throughput quantitative real time PCR (qPCR) platform.

Materials and Methods

The studies in Göttingen minipigs were designed and performed at Copenhagen University in Denmark. The Ossabaw minipig studies were designed and performed at Indiana University School of Medicine. The diet and design of the two studies are somewhat different, however in this study we characterised the gut microbiota in the two pig models separately.

Ethics statement

All the Ossabaw minipig experiments were approved by the Indiana University Animal Care and Use Committee (IACUC) (3645). The experiments meet the terms and the guidelines for the Care and Use of Laboratory Animals and the American Veterinary Medical Association Panel on Euthanasia. The Danish Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries approved the experiments involving the Göttingen minipigs.

Göttingen minipigs study

Female Göttingen minipigs (*n* = 14) that were ovariectomized (Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark) were subsequently housed at two years of age in facilities provided by the University of Copenhagen (Taastrup, Denmark) until they were euthanized at approximately 41-47 months of age. Of the 14 minipigs, seven were allocated as the obese group. The obese minipigs had been given therapeutic peptides for pharmacological studies prior to the obesity experiments but were subjected to a suitable washout period before the start of the present experiments. The lean group (*n* = 7) were fed restrictively with 150 g of minipig chow (Altromin 9023, Christian Petersen A/S, Gentofte, Denmark), twice a day consisting of 19% protein, 8% fat and 73% carbohydrates. The obese group (*n* = 7) were fed *ad libitum* Altromin 9033 (minipig chow, Christian Petersen A/S, Gentofte, Denmark) consisting of 25% protein, 11% fat, 64% carbohydrates. At approximately 122 days before the end of the experiment the animals were weighed biweekly and the body-fat composition was measured by dual energy x-ray absorptiometry scanning (DXA-scanning) (Hologic Explorer, Santax Medico, Aarhus, Denmark) at the end of experiment and the absolute mass of fat tissue was obtained by using the scanner software package (Cirera et al., 2012) and the absolute mass of fat tissue was obtained by using the scanner software package. All the animals were euthanized by pentobarbital. Colon and cecum samples with content were collected after the animals were euthanized. The samples were immediately frozen in liquid nitrogen and were subsequently stored at -80°C until further analyses.

Ossabaw minipigs study

The samples provided for this study were obtained from female Ossabaw minipigs that belong to Indiana University School of Medicine and Purdue University breeding colony (West Lafayette, IN, USA). The lean

Ossabaw minipigs (*n* = 4) received a daily diet (standard chow) with a total caloric content of 2200 kcal and the calories provided by macronutrients were: protein 18.4%, fat 10.5% and carbohydrates 71.0%. The obese group of minipigs (*n* = 4) received a high-energy diet of 4500-6000 kcal daily provided by: 16.3% protein, 42.9% fat and 40.8% carbohydrates (of which 20 % came from fructose). Both groups were housed individually at an age of six months, were fed the respective diets for a period of 8-10 months, and were euthanized at the end of feeding experiments at an age of 14-16 months. All the minipigs were euthanized by cardiectomy while under anaesthesia by a combination of intramuscular injections of tiletamine-zolazepam (5 mg kg^{-1}), xylazine (2.2 mg kg^{-1}) and isoflurane (5%). Samples from colon and terminal ileum both including content were collected after the animals were euthanized and were immediately frozen in liquid nitrogen and subsequently stored at -80° C until further analyses.

DNA extraction and purification

The DNA was extracted from colon and cecum digesta of lean (n = 7) and obese (n = 7) Göttingen minipigs and from colon and terminal ileum digesta of Ossabaw lean (n = 4) and obese (n = 4) minipigs. Digesta from colon, terminal ileum and cecum (200 mg each) was used for DNA extraction by Maxwell® 16 Cell DNA Purification Kit (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions with an additional bead beating step in order to disrupt the cell wall of Gram-positive bacteria.

PCR conditions

The DNA samples were used for amplification of the V5 region of the 16S rRNA gene. The PCR reactions were performed in duplicates of 49 µl per reaction consisting of 5 µL 5 X GoldTaq buffer (Applied Biosystems, Branchburg, NJ, USA), 20 µM of each forward and reverse primers, 2 µL dNTP (10 mM), 4 µL MgCl2 (25 mM), 0.5 µL AmpliTaq Gold® DNA Polymerase (5 U/ µL) (Applied Biosystems), 31.5 µL H2O and 2 µl DNA of 100 ng μl^{-1}. The PCR cycling conditions were an initial denaturing step of 94 °C for 6 min, followed by 30 cycles of 94°C for 45 s, 57°C for 45 s, 72°C for 45 s and a final extension step at 74°C for 10 min. The amplification was performed using the V5 universal primer set, forward primer 804F (5'- GGATTAGATACCCNGGTAGTC-3') and reverse primer 926R (5'- CCGTCAATTCCTTTRAGTTT-3') were performed in T3 Thermocycler (Biometra GmbH, D-37079 Göttingen, Germany). The quantity and quality of the resulting PCR products were then assessed on an Agilent 2100 Bioanalyzer using the Agilent DNA 1000 kit (Agilent Technologies, Waldbronn, Germany). The amplified DNA was purified for primers and detergents using a Qiagen MinElute PCR purification kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions and then pooled together and finally 3.6 ng of DNA were submitted to the National High-Throughput DNA Sequencing Centre at Copenhagen University, Denmark, for sequencing on an Illumina HiSeq™ 2000 platform.

Sequence analysis

The obtained sequences were sorted and normalized by BION-meta software with the taxonomic classification according to the Greengene Database [29]. In brief, the sequences were initially de-multiplexed according to the primer- and barcode sequences. Subsequently they were cleaned at both ends by removal of bases of a quality less than 96%. Identical sequences were further clustered and aligned into consensus sequences with a setting of 99.8% base quality. Consensus sequences of at least 40 nucleotides in length were further mapped into a table according to the individual barcodes. Finally, the consensus sequences were taxonomically classified against the Greengene SSU database using a word length of 8 and a match minimum of 30%. The top one percent of the obtained hits from the Greengene database was then used for taxonomical classification of the consensus sequences. The number of reads for each barcode was further normalized making it possible to do statistical analysis directly between individuals in the experiment. The Shannon-Weaver index of diversity (H') was further calculated manually to estimate the diversity of the bacteria at genus level from the obtained normalized sequences. Obtained data were statistically analysed in GraphPad prism version 5.00 for Windows (GraphPad software, San Diego, CA USA). Prior to analysis, data were log transformed for normalizations and analysed using one-way ANOVA or two-way ANOVA when appropriate. The Kruskal Wallis or Bonferroni's post hoc test was further used to test for differences among groups. P-values < 0.05 were considered significant. The abundances of bacteria in figures are represented as mean and error bars representing standard deviations.

Microbial identification by 48.48 dynamic array

High throughput qPCR was performed by a 48.48 Dynamic Array Integrated Fluidic Circuits (Fluidigm, CA, USA). This platform combines 24 primer sets in duplicates (48 primers) with 48 samples to run 2304 simultaneous qPCR reactions. In this chip, individual primer sets target the 16S rRNA gene DNA of different bacterial phylogenetic groups (Domain, Phyla, Class, Family, Genus and Species level) Hermann-Bank et al. (2012, in prep). Each sample consisted of 20 µmol l^{-1} forward and reverse primers, 1x Assay loading reagent (Fluidigm, PN85000746), 1x low EDTA TE buffer (AppliChem GmBH, Darmstadt Germany) and master mix consisting of; 20 X DNA binding dye sample loading reagent (Fluidigm, PN 100-0388), 20 X EvaGreen® DNA binding dye (Biotium, Heyward CA, USA,) and 2 x Taqman master mix (Applied Biosystems). The 16S rRNA gene DNA concentrations were optimized and equilibrated to a concentration of 50ng/ µl. In each run a non template control (NTC) was included to detect any contamination or non-specific amplification. A melting curve analysis was performed for quality check of amplification and the non-specific reactions were excluded. The obtained Ct values from the finally accepted values were subsequently exported to Microsoft Excel for further analysis. The relative proportion of bacteria representing each taxon was calculated based

on the Livak method. Hence, the relative quantifications of the PCR signal of the target 16S rRNA gene in the obese minipigs in each group was related to that of the lean minipigs which were considered to harbour the reference composition of the gut microbiota. Fold differences in the different bacterial groups were subsequently calculated by $2^{-\Delta\Delta Ct}$ [30]. All the statistical analysis were performed on GraphPad Prism version 5.00 for Windows (GraphPad software, San Diego, CA ,USA). The statistical analysis were performed by Mann-Whitney U test and significant differences were considered when $P < 0.05$.

Results

Phenotype of the Göttingen and Ossabaw minipigs

The lean Göttingen minipigs (n = 7) weighed 50.3 ± 1.6 kg when the animals were euthanized, while the obese Göttingen minipigs (n = 7) weighed 92.6 ± 5.2 kg. Approximately four weeks prior to euthanasia and at the time of DXA scanning, the body weight of the lean minipigs was 49 ± 1.6 kg with a body-fat percent of 26% (± 4) and the obese minipigs had an average body weight of 87 ± 5.3 kg with a body-fat percent of 42% (± 4). The difference between body-fat and body weight in lean and obese minipigs was significant ($P < 0.001$)[28]. There was no significant difference in blood triglycerides, total cholesterol and fasting blood glucose between lean and obese Göttingen minipigs. However there was a significant difference in fasting blood insulin levels between lean and obese Göttingen minipigs ($P < 0.01$) (Table 1).

The lean Ossabaw minipigs (n = 4) at six months of age weighed 43.3 ± 6.6 kg and by the end of the experiment, at an age of 16 months they weighed 60.4±7.5 kg. At six months of age the obese Ossabaw minipigs (n = 4) weighed 42.8 ±2.4 kg and at the end of experiments they weighed 98.3 ±2.9 kg. The difference between body weight in lean and obese Ossabaw minipigs was significant ($P < 0.01$). The obese Ossabaw minipigs displayed MetS with total blood cholesterol and triglycerides higher than the lean Ossabaw minipigs ($P<0.005$ and $P<0.01$, respectively). Even though the fasting blood glucose levels showed a trend toward being higher in obese than in lean minipigs and a similar trend was observed in blood insulin levels in obese (34.7 pmol l^{-1}) and in lean (15 pmol l^{-1}) the differences were not significant ($P<0.1$) [23,31].

Sequence analysis

Obtained sequence files were analysed using the Bion software. A total of 603,926,924 reads were obtained from the Illumina sequencing. After de-multiplexing according to primer and barcode sequences a total of approximately 469 mill remaining reads were 3' and 5' trimmed according to base quality. Sequences below a quality of 96 % were removed. The number of sequences used for taxonomical classification was 339,826,026 reads. Out of these 99.9% of the sequences were classified according to the Greengene SSU database.

I. Characterization of the intestinal microbiota in Göttingen minipigs

In Göttingen minipigs the five most abundant phyla constituted 98.1% of all the obtained phyla (Figure 1A). The most abundant sequences obtained at phyla level were the Firmicutes consisting of 49.3% of all the normalized reads and the second most abundant phyla, the Bacteroidetes constituted 38.3% of all the normalized reads. Spirochaetes, Tenericutes and Proteobacteria constituted 6%, 3.8% and 0.6 % of all the normalized reads, respectively. The diversity analysis of colonic and cecal microbiota from the obtained sequences at genus level showed no difference between lean and obese Göttingen minipigs.

At phyla level, the abundance of Firmicutes was higher in cecal microbiota of lean minipigs than the obese minipigs ($P < 0.006$) while no significant difference was observed in Bacteroidetes between the two groups of minipigs (Figure 1B).

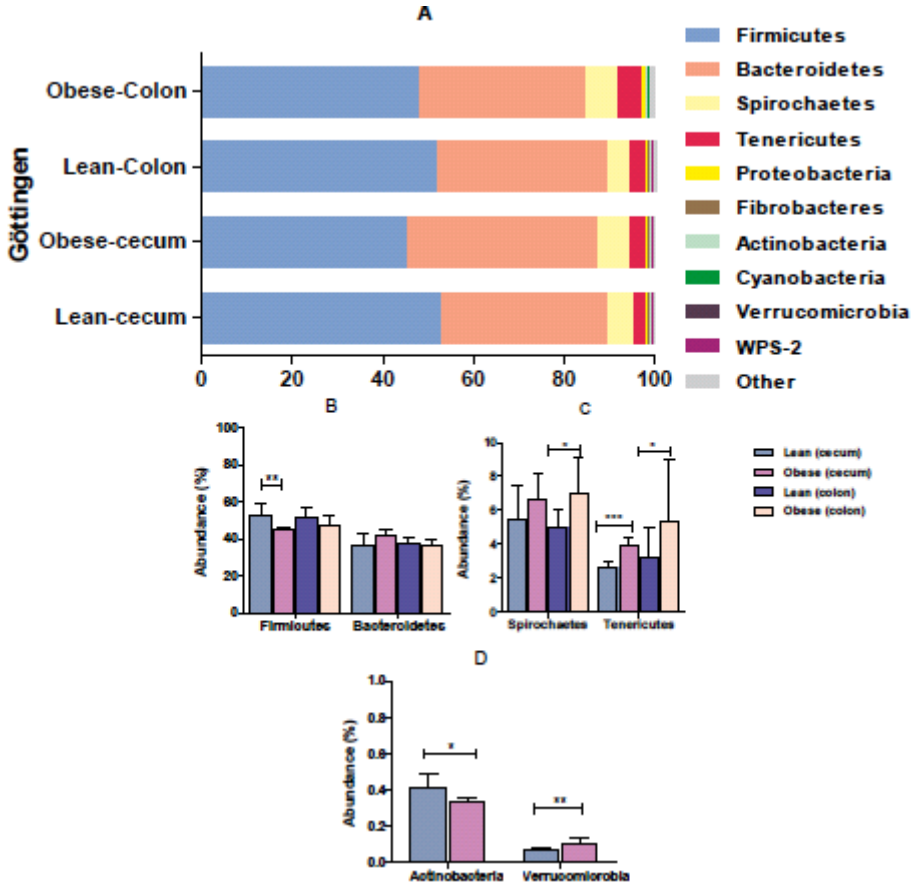

Figure 1. Abundance of phyla in colonic and cecal microbiota of lean and obese Göttingen minipigs. (A-D) Deep sequencing analysis in different genera in colon and cecum of lean and obese minipigs. The percentages are calculated from all identified sequences in each group.

The abundance of Spirochaetes was higher in colonic microbiota of obese minipigs ($P<0.03$) as compared to lean minipigs. The abundance of Tenericutes was higher in both cecal and colonic microbiota of obese minipigs as compared to lean minipigs ($P < 0.004$, $P = 0.04$) (Figure 1 C). A higher abundance of Actinobacteria was observed in lean cecal microbiota of Göttingen minipigs ($P = 0.01$). In other phyla, there

was a significant difference in the abundance of Verricomicrobia between lean and obese minipigs' cecum (P = 0.005), with the obese cecum containing a higher abundance of the bacteria belonging to this phyla (Figure 1 D). In both lean and obese minipigs, the cecal microbiota consisted mainly of Firmicutes while the colonic microbiota had a higher abundance of Bacteroidetes. The ratio of Firmicutes to Bacteroidetes was significantly higher in cecal (P< 0.0005) and colon (P<0.0001) microbiota of lean Göttingen minipigs. In obese Göttingen minipigs only cecal microbiota had higher ratio of Firmicutes to Bacteroidetes (P<0.0006).
At family level, the 15 most abundant families of bacteria constituted 94.7% of all bacteria and unknown Bacteroidales, Ruminococcaceae, Lachnospiraceae, Prevotellaceae and unknown Clostridiales were the most abundant families (Figure 2 A).

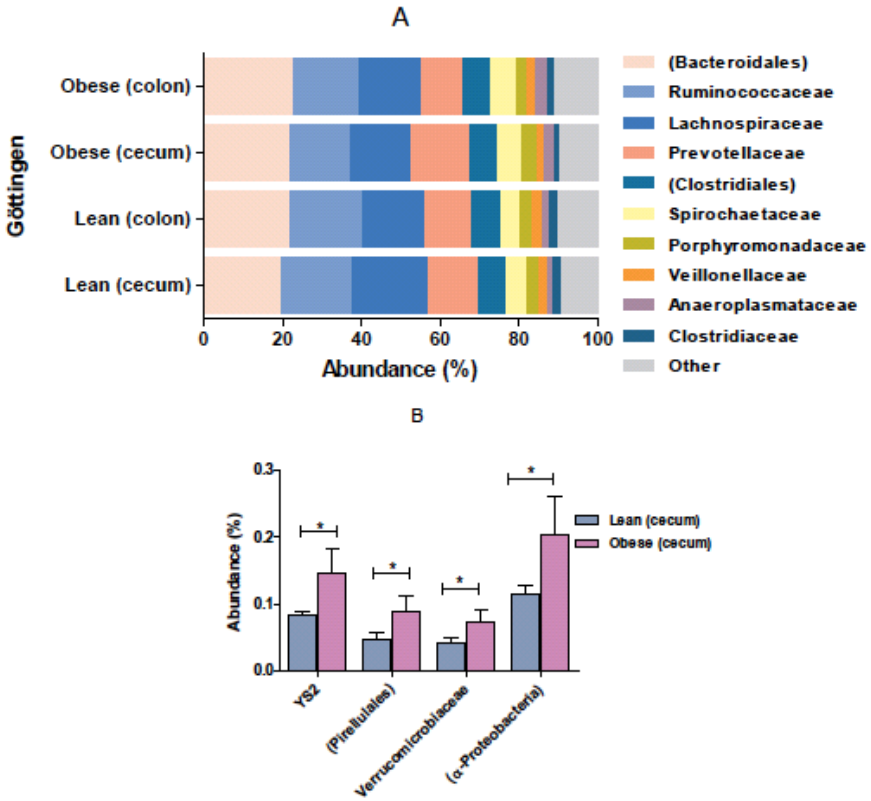

Figure 2 Abundance of different bacterial family in colonic and cecal microbiota of lean and obese Göttingen minipigs. (A-B) Sequencing analysis in different genera in colon and cecum of lean and obese minipigs. The percentages are calculated from all identified sequences in each group. Parentheses indicate unknown Family.

There was no difference in the microbiota in neither colon nor cecum between lean and obese minipigs in abundance of *Lactobacillaceae* and *Enterobacteriaceae.* A higher abundance of *Streptococcaceae* (P = 0.02) and *Bifidobacteriaceae* (P = 0.04) was observed in cecum samples from lean pigs. The abundance of *Methanobacteriaceae* was higher in colonic microbiota of obese pigs than the lean pigs (P < 0.01). There were several differences between lean and obese cecal microbiota in bacteria belonging to different families (Figure 2 B).

At genus level, the 50 most predominant genera consisted of 97.5% of the entire genera represented from all the obtained reads (normalized reads). The most predominant genera were unknown genera belonging to order *Bacteroidales (Bacteroidales), Unknown (Ruminococcaceae), Unknown (Lachnospiraceae*), constituting 21.2%, 11.1% and 9.5% of all the normalized reads obtained at genus level, respectively (Figure 3 A). The genus *Prevotella* constituted 9.4% of all the normalized reads. Several small unknown genera had a higher abundance in cecum of obese minipigs (Figure 3 B and C) while there were no significant differences between the two group in colonic microbiota. Several genus belonging the phylum Tenericutes were significantly higher in the obese cecal microbiota such as unknown genus (Anaeroplasmataceae) ($P < 0.001$), RF39 ($P < 0.01$), *unknown (Erysipelotrichaceae)* ($P < 0.001$) and *unknown (Tenericutes)* ($P < 0.001$) (Figure 3 B). Of other bacteria in obese cecal microbiota with significant difference were *Sphaerochaeta* ($P < 0.05$), *unknown (Alphaproteobacteria)* ($P < 0.001$), *YS2 (Cyanobacteria)* (<0.001), *Unknown (Pirellulales)* ($P < 0.001$). *Methanobrevibacter* ($P < 0.01$) and *unknown β-proteobacteria (Alcaligenaceae)* had a higher abundance in lean cecal microbiota (Figure 3 C).

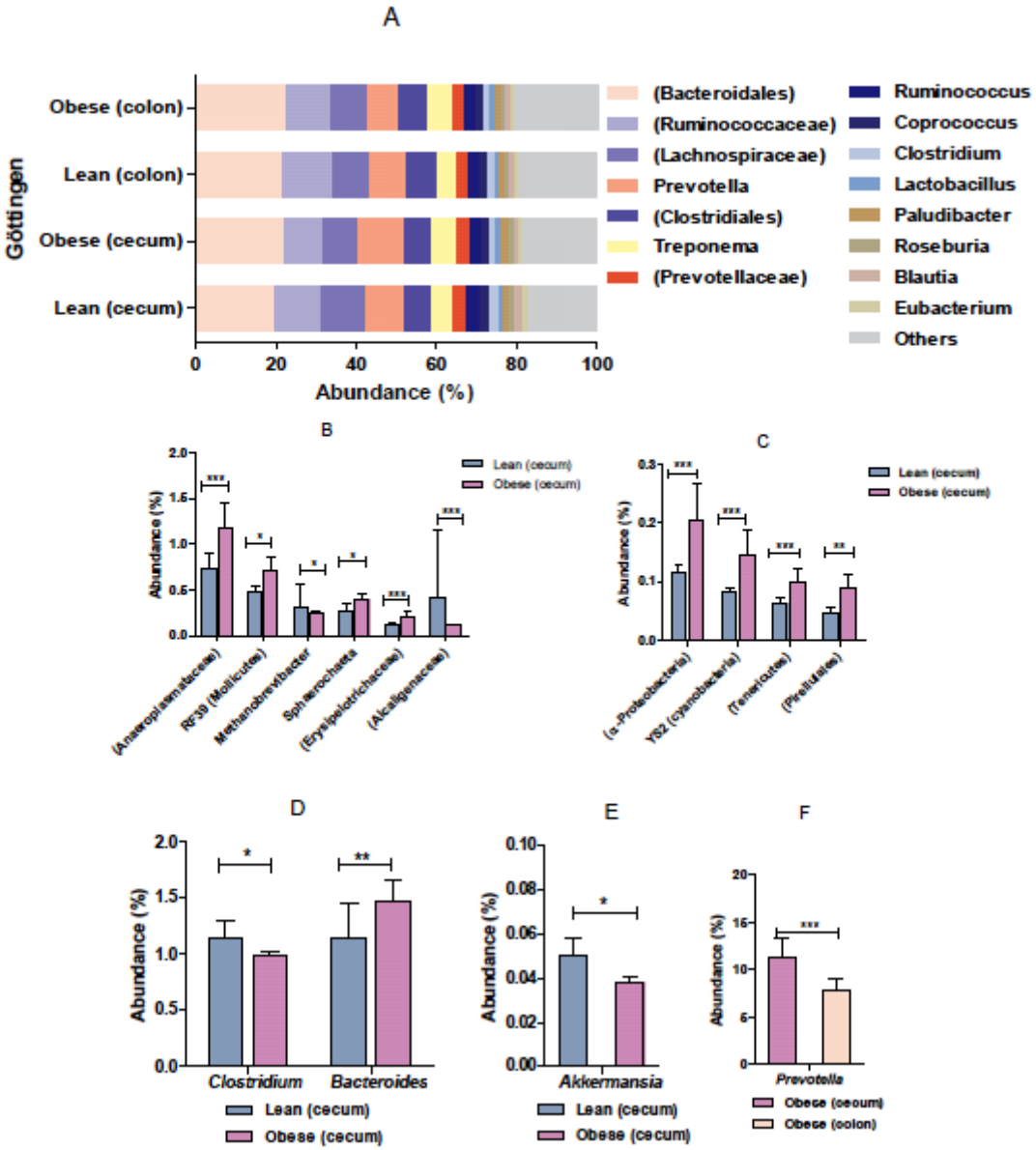

Figure 3 Abundance of different genera in colonic and cecal microbiota of lean and obese Göttingen minipigs. (A-F) Deep sequencing analysis in different genera in colon and cecum of lean and obese Göttingen minipigs. The presented percentages are calculated from all identified sequences in each group. Parentheses indicates unknown genus.

The lean minipigs had a higher abundance of *Clostridium* in cecum as compared to the obese minipigs (P = 0.01) (Figure 3 D) and the cecal microbiota of obese minipigs had a higher abundance of *Bacteroides* compared to lean minipigs (P = 0.01) (Figure 3 D). A higher abundance of the genus *Akkermensia* was observed in cecum of lean minipigs as compared to obese minipigs (P < 0.01) (Figure 3 E). The colonic and cecal microbiota of obese minipigs was different in abundance of *Prevotella* which were higher in obese cecal microbiota (P < 0.0001) (Figure 3 F).

II. Characterization of the intestinal microbiota in Ossabaw minipigs

To characterise the intestinal microbiota of lean and obese Ossabaw minipigs, the colonic and ileal microbiota were sequenced. Overall, the ten most abundant phyla constituted 99.7% of all the phyla obtained from Illumina sequencing (Figure 4 A). The statistical analyses were performed from the six most abundant phyla, constituting 98.4% of all the phyla obtained. These phyla were the Firmicutes consisting of 54.57% of all the normalized reads followed by Bacteroidetes with 27.34% of all the normalized reads. The next most abundant phyla were Proteobacteria (8.4%), Spirochaetes (3.6%), Fusobacteria (3%), Actinobacteria (1.3%) and Tenericutes (0.5%), respectively. The diversity analysis of colonic and ileal microbiota from the obtained sequences at genus level showed no difference between lean and obese minipigs.

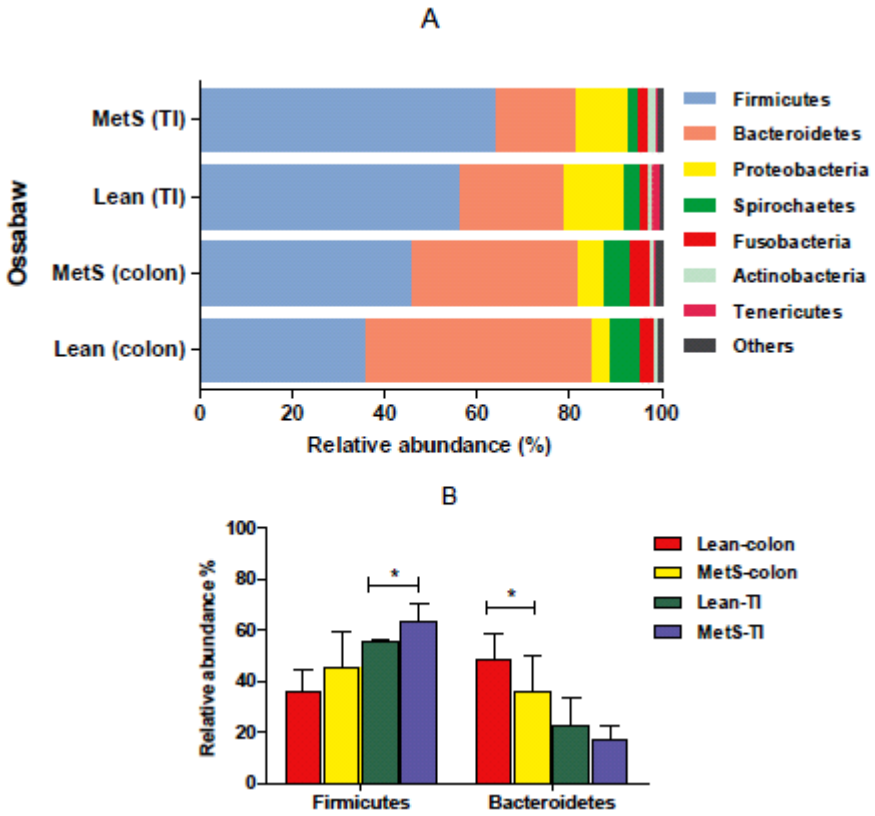

Figure 4 Microbial profiles of colon vary between lean and obese (MetS) Ossabaw minipigs. Sequencing analysis on selected phyla (A, B) in terminal ileum (TI) and colon of lean minipigs and obese minipigs. The percentages are calculated from all identified sequences in each group.

A higher relative abundance of Firmicutes and a relative lower abundance of Bacteroidetes were observed in both colon and ileal microbiota in obese minipigs as compared to lean minipigs ($P < 0.01$) (Figure 4 B). The abundance of Bacteroidetes and Firmicutes were reversed in lean minipigs, with lean minipigs' microbiota in colon consisting of 48.7% Bacteroidetes and 35.8% Firmicutes, while the obese minipigs had 45.7% Firmicutes and 35.9% Bacteroidetes in their colonic microbiota. Within the lean minipigs a higher relative abundance of Bacteroidetes was observed in colon compared to terminal ileum ($P = 0.002$). There was a

trend towards higher abundances of Proteobacteria and Spirochaetes in obese minipigs' colon as compared to lean.

At class level the 10 most abundant classes constituted 98.2% of all the classes obtained from the normalized reads. Statistical analysis was performed on *Clostridia*, *Bacteroidia*, *Bacilli*, *α-*, *β-*, *δ-*, *γ,ε-Proteobacteria*, *Mollicutes* and *Actinobacteria*. There was a higher abundance of *Clostridia* in microbiota from colon in obese minipigs as compared to lean minipigs colonic microbiota ($P < 0.04$) and a higher abundance of *Bacteroidia* in lean colon with 48.5% (±9.8) of all bacteria as compared to colon samples from obese minipigs (35.5% (±14)). The class *Bacilli* belongs to the phyla *Firmicutes*, were highest in abundance in terminal ileum of obese minipigs ($P < 0.0001$). Overall the abundance of *β-Proteobacteria* was higher in the microbiota from terminal ileum of both lean and obese minipigs compared to colon ($P = 0.02$).
The families Prevotellaceae, Clostridiaceae and Lachnospiraceae together constituting 39.2% of all the normalized reads obtained at family level (Figure 5A). Prevotellaceae was more abundant in lean minipigs than in obese minipigs with a mean relative abundance of 30.8% and 19.4%, respectively (Figure 5 B). There was a higher abundance of Lactobacillaceae in terminal ileum of lean minipigs ($P = 0.01$) compared to obese minipigs. The abundance of Streptococcaceae was highest in terminal ileum of lean minipigs ($P < 0.003$) and generally higher in terminal ileum than colon (Figure 5 A).

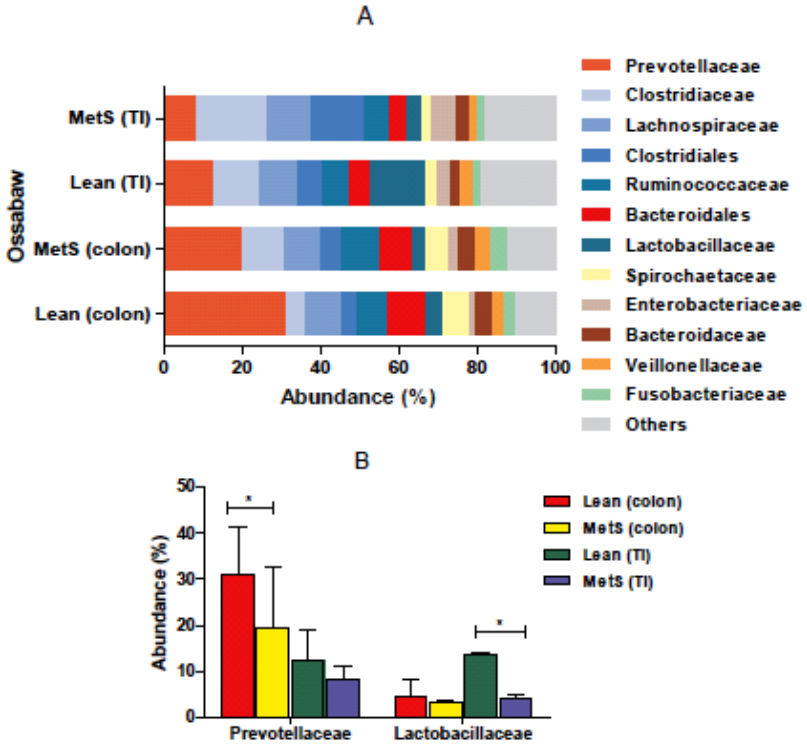

Figure 5 Different microbial profiles in microbiota of lean (Lean) and obese Ossabaw minipigs (MetS) at family level. Sequencing analysis in different phyla (A, B) in terminal ileum (TI) and colon of lean and obese Ossabaw minipigs. The percentages are calculated from all identified sequences in each group. Parentheses indicate unknown Family.

At genus level, the 50 most predominant genera consisted of 93.1% of the entire genera present in all obtained normalized reads. The two most abundant genera were Prevotella and Clostridium both consisting of 16.1% and 11.7% of all the normalized reads, respectively (Figure 6 A). In lean minipigs, the colonic microbiota had a higher abundance of Prevotella ($P < 0.01$) with a mean relative abundance of 28% (±5.5) while the obese minipigs had a mean relative abundance of 18.3% (±4.0) (Figure 6 B). However, Clostridium was more abundant in colonic microbiota of obese minipigs ($P < 0.01$) than lean minipigs (Figure 6 B).

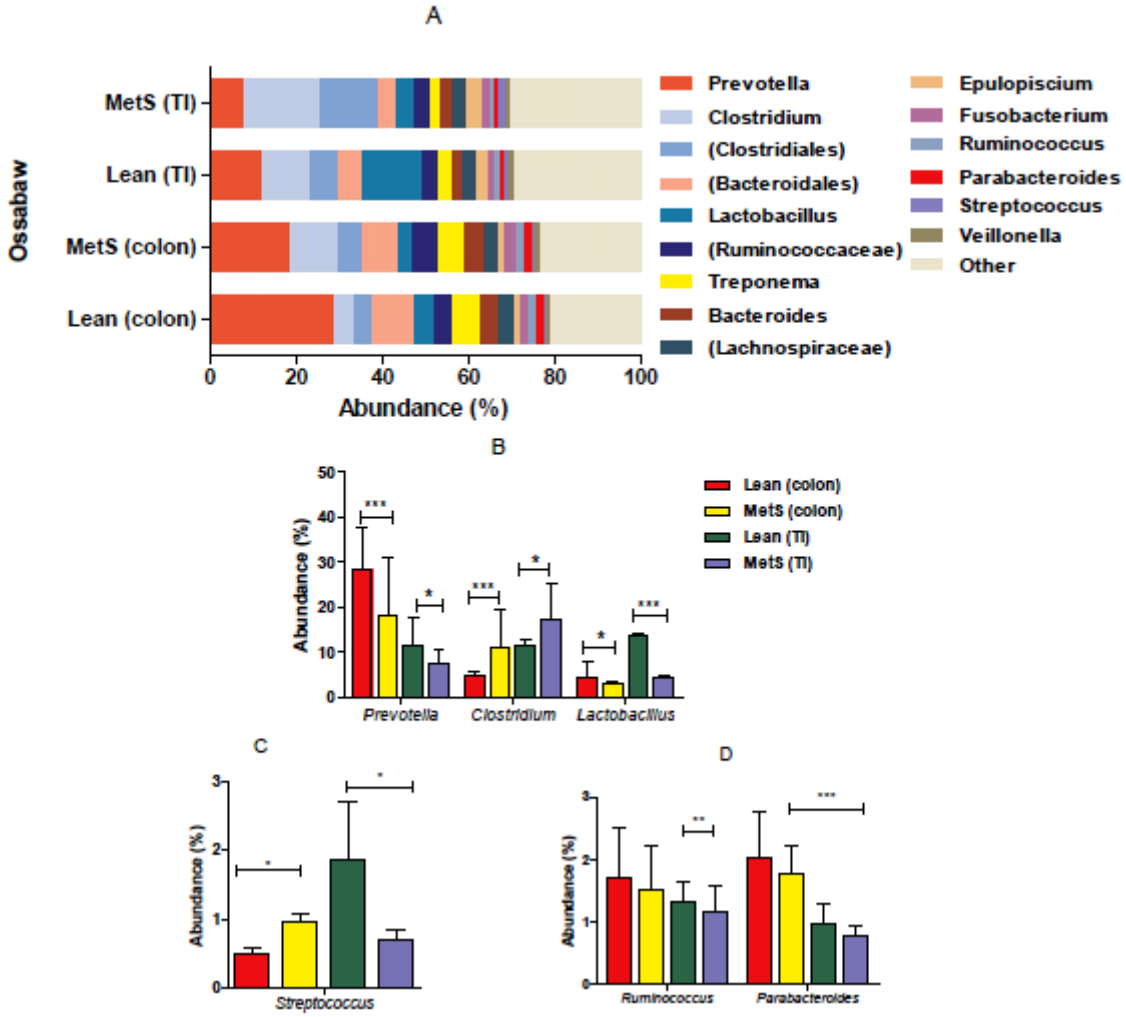

Figure 6 Abundance of different genera in colonic and ileal microbiota of lean and obese (MetS) Ossabaw minipigs. (A-D) Sequencing analysis in different genera in terminal ileum (TI) and colon of lean and obese minipigs. The percentages are calculated from all identified sequences in each group.

Lactobacillus, the fifth most abundant genus obtained from all the normalized reads, constituted 5% of the entire genera. There was a significant difference between the abundance of *Lactobacillus* within the microbiota from colon of lean minipigs having a higher abundance than to the microbiota from obese minipigs ($P < 0.0001$) (Figure 6 B). The abundance of two lactate producing bacteria belonging to *Streptococcus* were higher in obese minipigs than in lean minipigs ($P = 0.03$; $P = 0.02$, respectively) (Figure 6 C). Overall, the abundance of *Streptococcus* was higher in terminal ileum than colon in both lean and obese group ($P < 0.01$). There were several differences in bacteria belonging to different genera between the

microbiota in colon and terminal ileum in both lean and obese minipigs. Generally *Parabacteroides* was more abundant in colon than terminall ileum (Figure 6 D).

III. Fold-differences between lean and obese minipigs in selected groups of bacteria

The obtained results from high throughput qPCR show several differences between lean and obese minipigs in both Göttingen minipigs and Ossabaw minipigs. There were many groups of bacteria that were not detectable by this qPCR system, such as *Verrucomicrobia*, *Bifidobacteriaceae*, *Cl. cluster I*, *δ-*, *β-*, *γ-Proteobacteria*, *Enterococcus* and *E.coli*. *Actinobacteria* had a 5 fold higher relative abundance in colon and terminal ileum of obese Ossabaw minipigs than lean minipigs, however this was not significant. A 4 fold higher abundance of bacteria belonging to the genus *Bacteroides* was observed in terminal ileum and colon of obese Ossabaw minipigs (Table 1).

Table 1 Fold-differences between lean and obese minipigs in relative abundance of bacteria in different phylogenetic groups in cecal and colonic microbiota of obese Göttingen minipigs and in ileal and colonic microbiota of obese Ossabaw minipigs, estimated by qPCR dynamic array. (* indicates values with significant difference)

	Göttingen-Cecum	Göttingen-Colon	Ossabaw-TI	Ossabaw-Colon
Firmicutes	1	N.D.	N.D.	N.D.
Bacteroidetes	<2	**7.6**	<2	1
Actinobacteria	<1	<1	**5.0**	**5.5**
Fusobacteria	N.D.	1	N.D.	N.D.
Verrucomicrobia	N.D.	N.D.	N.D.	N.D.
Spirocheates	**3.3**	N.D.	N.D.	N.D.
Bacilli	N.D.	N.D.	N.D.	N.D.
B- and γ-Proteobacteria	N.D.	N.D.	N.D.	N.D.
δ-Proteobacteria	N.D.	N.D.	N.D.	N.D.
ε-Proteobacteria	N.D.	2	N.D.	N.D.
Lactobacillaceae	<1	<1	1	<1
Streptoccocceae	<1	<1	N.D.	N.D.
Cl.Cluster I	N.D.	N.D.	N.D.	N.D.
Cl. Cluster IV	<1	N.D.	<2	<1
Cl.Cluster XIV	<1	**7.6***	<1	<1
Bifidobacteriaceae	N.D.	N.D.	N.D.	N.D.
Enterobacteriaceae	N.D.	N.D.	N.D.	N.D.
Enterococcus	N.D.	N.D.	N.D.	N.D.
Bacteroides	N.D.	N.D.	**4.6**	**4.3**
Cl. Perfringens	N.D.	**3.5**	N.D.	N.D.
E.coli	N.D.	N.D.	N.D.	N.D.

In the obese Göttingen colon microbiota, there was a 7.8 fold higher abundance of *Bacteroidetes* as than in the lean minipigs; however this was not significant (Table 1). The only significant difference was found in relative abundance of bacteria belonging to the *Cl. cluster XIV* family, with a 7.6 fold higher abundance in colon of obese Göttingen minipigs as compared to the lean group ($P < 0.0001$).

Discussion

In this study we aimed to characterise the composition of the intestinal microbiota of two pig models of obesity and the effect of diet and adiposity on gut microbial community in these pigs. Göttingen minipigs have been used as obesity models and displayed minor abnormalities in glucose tolerance and insulin sensitivity (Larsen et al., 2002), however the metabolic changes in lean and obese Göttingen minipigs on the diets used in the present study are not well described. Ossabaw minipigs have been used as animal models in studies of obesity related metabolic disorders such as cardiovascular disease and are predisposed to insulin resistance in response to obesity and high-fat feeding, which is unique in Ossabaw minipigs as opposed to other animal models (Neeb et al., 2010). Lee *et al*. (Lee et al., 2009b) reported several differences in blood parameters such as cholesterol and triglycerides between lean and obese Ossabaw minipigs. The Ossabaw minipigs used in our study displayed metabolic syndrome with abdominal and visceral adiposity, high blood triglyceride levels and high total blood cholesterol (Lee et al., 2009b). To our knowledge the Ossabaw minipigs intestinal microbiota has not been characterised before and this is the first study that investigated the relation between obesity and intestinal microbial community in Ossabaw minipigs by16S rRNA gene sequencing using the Illumina technology.

Here we report that the gut microbiota in relation to obesity was different between lean and obese Ossabaw minipigs. Our data suggest that obesity or HFD have affected the gut microbiota of obese Ossabaw minipigs since they had an overall different gut microbial community as compared to their lean counterparts. HFD has previously been shown to affect the gut microbiota both in obesity and independent of the obese state (Hildebrandt et al., 2009) and ingestion of HFD has been implicated in causing metabolic disorders observed in obese pigs and mice (Larsen et al., 2002;Cani et al., 2008). In our study the obese Ossabaw minipigs received HFD which could also have caused the differences observed between lean and obese Ossabaw minipigs. At phyla level the only significant differences between the lean and obese Ossabaw minipigs were observed in abundance of *Firmicutes* and *Bacteroidetes* with higher abundance of *Firmicutes* in obese minipigs and higher abundance of *Bacteroidetes* in lean minipigs. Our results are in agreement with studies in obese humans (Ley et al., 2006b), mice (Ley et al., 2005) and pigs (Guo et al., 2008a) that displayed only obesity, while the results contradicts several human studies in subject with T2D (Schwiertz et al., 2009;Larsen et al., 2010). However at lower taxonomic levels, more differences were observed between lean and obese Ossabaw minipigs. A marked difference was observed in the abundance of the *Prevotellaceae* and *Lactobacillaceae*, especially genera *Prevotella* and *Lactobacillus*, which had higher abundance in lean Ossabaw minipigs while these results are opposite to the findings reported previously with higher abundance of *Prevotella* in diabetic humans and mice (Larsen et al., 2010;Serino et al., 2012b). Our results are in agreement with those reported by Turnbaugh et al.(Turnbaugh et al., 2006), indicating that the state

of obesity shapes the gut microbiota. However diet is also a factor that may have caused the changes we observed here. Furthermore, the abundance of *Lactobacillus* was significantly lower in colon of obese Ossabaw minipigs as compared to lean minipigs and this result has been shown before in mice fed a HFD (Lam et al., 2012) implicating diet as another factor that shapes the intestinal microbiota. HFD has been implicated in changing the gut permeability by modulating the gut microbiota. A positive correlation has been observed between higher abundances of *Lactobacillus* and colonic transepithelial resistance (Lam et al., 2012). The lean Ossabaw minipigs had a higher abundance of *Lactobacillus* in their colonic microbiota compared to obese and do not display metabolic dysfunction as compared to obese Ossabaw minipigs. The low abundance of *Lactobacillus* observed in obese Ossabaw minipigs could have caused changes in their intestinal permeability; however the intestinal permeability was not measured in our study. Increased gut permeability has been postulated to be related to metabolic endotoxemia, causing metabolic dysfunctions such as T2D. In case of *Lactobacillus*, the gut microbiota may have contributed in shaping the metabolic profile observed in obese Ossabaw minipigs by changing the gut permeability. Together our results suggest that the obese Ossabaw minipigs' intestinal microbial community display a profile that resembles the intestinal microbiota observed in obese mice and humans in response to HFD.

We also attempted to characterise the Göttingen minipigs cecal and colonic microbiota in relation to obesity which was induced by overeating and not high-fat/high-energy diet. In the cecum microbiota of Göttingen minipigs there were several differences related to their metabolic state, while no differences were seen in the microbiota in colon. Interestingly the ratio of *Firmicutes* to *Bacteroidetes* was opposite to the findings in Ossabaw minipigs since lean Göttingen minipigs had a higher abundance of *Firmicutes* relative to *Bacteroidetes.* These results are however in agreement with the findings in diabetic mice and humans (Serino et al., 2012b;Larsen et al., 2010). One of the interesting findings was the higher abundance of bacteria belonging to phyla *Tenericutes* (class Mollicutes) in obese Göttingen minipigs compared to their lean counterparts. The bacteria belonging to this phylum have previously been implicated to be increased in diet-induced obese mice (Turnbaugh et al., 2008). Noteworthy was the differences observed in *Bacteroides* between lean and obese Göttingen minipigs, with higher abundances in obese minipigs which has previously been reported to be connected to gut microbiota of T2D in humans (Larsen et al., 2010). Shortly, the cecal microbiota of obese Göttingen minipigs was different from that of lean minipigs with higher abundance of bacteria belonging to phyla *Bacteroidetes*, *Spirochaetes*, *Tenericutes* and *Verrucomicrobia*, indicating a specific gut microbial profile in obese Göttingen minipigs. We also identified genera with higher abundances in lean Göttingen minipigs, namely *Akkermensia* and *Methanovibrobact*er. Species belonging to *Akkermensia* have been shown to play a protective role in the gut microbiota of mice against autoimmune disease such as type 1 diabetes (Hansen et al., 2012) and species belonging to the *Methanovibribacter*, have been connected to a lean phenotype (Armougom et al., 2009). Recent studies have shown that the species

Methanovibribacter smithii co-occurs with several other species such as species in the genus *Clostridia*. Together, these findings can be used to approach the obesity-related gut microbiota by exploring how the microbial community co-occur in periods of high energy feed and how this diet affects the community and its' stability over a period of time. Such information can be used to develop probiotic strains to change the gut microbial community into a composition that protects against increased gut permeability, low grade inflammation and obesity and the related metabolic disorders

According to our data the gut microbiota of obese Ossabaw minipigs intestinal microbiota displays the characteristic of obese gut microbiota and not MetS. However it must be mentioned that the data in Ossabaw minipigs is based on a small number of animals and therefore a larger population would provide a better understanding of the Ossabaw minipig gut microbial community. Obese Göttingen minipigs display a specific gut microbiota that has previously been connected to metabolic disorders such as T2D. Even though obese Göttingen minipigs do not display metabolic disorders in proportions to that of obese Ossabaw minipigs, their gut microbial profile have similarities to those found previously in T2D mice. Therefore Göttingen minipigs have great potential for being used in obesity related gut microbial studies. Here it seems that certain group of bacteria flourish best under HFD conditions such as *Firmicutes* as observed in Ossabaw minipigs, while others such as the genus *Bacteroides* flourished due to obesity under other diet condition as observed in obese Göttingen minipigs.

In conclusion, our data suggest diet as an important factor that shapes the gut microbial community as shown in Ossabaw minipigs. Obesity is connected to radical changes in several bacterial groups as observed in obese Göttingen minipigs. Here we show that both Ossabaw and Göttingen minipigs may be useful animal models in studying gut microbiota and provide a better understanding of obesity and metabolic syndrome and the relationship to the gut microbiota. Together, these findings can be used to approach the obesity-related gut microbiota to develop probiotic strains.

Author's disclosure statement

RP carried out the sampling from Göttingen minipigs and the experimental work, data and statistical analysis and wrote the manuscript. MB contributed to the designing of experiments and editing of the manuscript. HCI contributed to sequence data analysis and editing of the manuscript. SCS, SGM and BØC performed the animal experiments regarding Göttingen minipigs. MA and MS performed the animal experiments regarding Ossabaw minipigs. NC contributed with the Bion software used for sequence analysis. All authors have read and approved the final manuscript. All the authors have contributed to editing of the manuscript. All the authors declare no competing interests.

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7 Discussion

In this section the methodological aspects of the study that has not been discussed in the article, will be discussed. The biological aspects of the study in the different animal studies that were not discussed in the articles will be discussed here as well.

7.1 General discussion

In this study different methods have been used to obtain a profile of the composition of the gut microbiota in three different pig models of obesity with different genetic background and diet. Therefore, the gut microbiota of the three pig models cannot be compared or conclusions drawn accordingly without considering these factors. However, the three pig models provide an insight into the gut microbial community in these animals and the effect of diet and obesity on the bacterial communities that reside in their intestinal tract. Not only were the three experimental designs different but also the methods used to study the composition of their gut microbiota were different as well.

In the Nutriomics study of cloned lean and obese pigs, T-RFLP was performed to obtain a profile of the gut microbial community in faecal samples obtained biweekly. T-RFLP is suitable when analyzing and comparing microbial communities. Furthermore, T-RFLP can be used to monitor changes in the composition and structure of a microbial community. However, identification of bacteria is not precise by *in silico* analysis. This is not only due to bacterial sequences that are yet to be identified and the incomplete sequence databases, but also the universal primer 8f, is not as specific and is not as universal as it should be, therefore may not have covered all the bacteria (Schütte et al., 2008). The *in silico* analysis was performed by online programs such as MICA and RDP and as expected many T-RFs were not identified and therefore the bacterial identifications were not possible. Most of the results obtained by T-RFLP were used for studying the bacterial diversity and comparing the gut microbial community from the obtained T-RFs in all the animals. Therefore use of clone libraries is necessary in bacterial identification. These methodological limitations made it necessary to use other methods such as qPCR. In this thesis qPCR was performed to identify the relative abundance of *Bacteroidetes* and *Firmicutes* (**Paper I paper II**).

The results obtained from the qPCR runs were satisfactory, although it must be mentioned that some things could have been done differently. A standard curve was constructed from nano-gram genomic DNA for each run, however this standard curve would have given a more precise bacterial copy number and absolute values if it was constructed from known plasmids containing cloned target sequences and subsequently the plasmid templates corresponding to copy numbers of target rRNA gene sequences. Nonetheless, in comparing the abundance of two bacterial phyla in the gut microbiota from two

treatment groups (e.g. lean vs. diet-induced obese), to identify changes in these bacterial groups, absolute quantifications may not be necessary (Larsen et al., 2011).

Another qPCR method was used for identification of bacteria at different phylogenetic levels. This high-throughput qPCR was recently designed (Herman-Bank et al. 2012, *in prep*) and at the time it was used in this thesis. This method has undergone several optimizations which were only known to the designer of the platform. Several difficulties were experienced using this qPCR platform, firstly the data obtained from each chip was very low in output and there were many unspecific amplifications as well as lack of amplification even by the universal primers as shown in Figure 12. Secondly there were no standard curves in order to quantify the Ct values relatively and PCR efficiency was unknown. At the time the chip was used for the studies in this paper, a standard curve could not be constructed due to the limited knowledge about the technique. Instead the Ct values were relatively quantified against the universal primer. Therefore the data that was detectable and could be analyzed showed a fold-change or fold-difference between two different groups of animals Finally, sequencing by Illumina may therefore present a better alternative to the above mentioned methods, providing large amounts of data and giving a better picture of a gut microbiota as compared to other techniques.

7.1.1 Host genome and gut microbiota: The cloned pig as animal model

The hypothesis in the cloning study was that cloning of pigs would reduce inter-individual variations and thereby provide a unique animal model. Obesity being a multi factorial disease would need a study model with minimal genetic variation between test subjects; therefore animals with identical genotype may provide a good model. Furthermore, the cloned pigs were to provide a model for obesity related gut microbiota studies. However, in **Paper I** it was reported that the obese cloned pigs did not have less inter-individual variation as compared to their non-cloned counterparts, at least in regard to gut microbiota study. These results are in line with what was found by other groups using the same animals for other studies (Clausen et al., 2011;Christensen et al., 2012). Nevertheless, in **Paper II,** from the profiling of gut microbial community by T-RFLP, it was found that the lean cloned pigs clustered differently than their non-cloned counterparts (Figure 2, **Paper II**). This was observed by multivariate analysis through PCA.

Through qPCR analysis we observed less variation in both lean and obese cloned pigs with regard to the relative abundance of *Bacteroidetes* in both colon and terminal ileum (figure 4b and 5a in **Paper II**). This was not expected as we had not observed less variation in the cloned pigs' in neither *Firmicutes* nor gut microbial profile as evaluated by PCA and diversity analysis of T-RFs. This was not discussed in **Paper II** mainly because of the many factors that may have affected the results observed here. However, other studies have shown large inter-individual variations between faecal communities which can explain why this was observed in phyla *Bacteroidetes* and not *Firmicutes* (Zoetendal et al., 1998).

The results presented in **Paper II** are from colon content as opposed to faecal content and based on all obese cloned pigs (*n*=9), while qPCR results from faecal samples presented in **Paper I** are based on only five cloned pigs. As both cloned and non-cloned pigs were housed individually upon start of diet-intervention studies and were fed the same diet, these two factors can be excluded. Studies on human monozygotic twins have revealed similar degree of variation in the gut microbiota between the twin siblings which have been argued to be due to early environmental exposures which shapes the structure of adult individuals (Turnbaugh et al., 2009a). The early environmental factors could be argued as one factor that may have affected the results observed here. Other studies on the same pigs have however shown differences between cloned and non-cloned pigs. Rødgaard et al. (2012) showed that the obese cloned pigs had even higher inter-individual variations in subset of serum proteins than non-cloned pigs and cloning affected the expression of several innate immune genes. Furthermore, it was shown that the number of genes that respond to obesity was decreased in cloned pigs as compared to the non-cloned pigs. These studies were performed on different adipose tissue and lever but not on intestinal tissue (Rødgaard et al., 2012). If it is assumed that the same changes in innate immune genes observed in cloned pigs apply for the intestinal immune genes as well, this could explain the differences observed between cloned and non-cloned pigs. In fact recent studies have revealed that the microbial ecology of the gut is affected by host genotype and innate immune system (Walter and Ley, 2011). Nonetheless, the results in this thesis may reflect the genetic relatedness among the cloned pigs and thereby a less variation in the abundance of *Bacteroidetes*. The reason to why this was not observed in other bacterial groups is yet to be discovered.

7.1.2 Diet and gut microbiota

Diet has been shown to have an effect on composition of the gut microbiota in humans (Jumpertz et al., 2011) and mice (Hildebrandt et al., 2009). The diet for the animals in the Nutriomics project consisted of HF/HE diet which caused obesity in all pigs when fed *ad libitum*. The cloned pigs in the *ad libitum* group however did not gain as much weight as the non-cloned pigs even though they had the same weight at the start of the diet-intervention study. This was shown to be mainly due to smaller amounts of food intake as compared to non-cloned pigs (Christensen et al., 2012). Nevertheless the gut microbiota from cloned pigs showed a change in composition from lean to obese state with a progressive increase in *Firmicutes* as shown in other time-line studies (Murphy et al., 2010)(**Paper I**). As expected, the relative abundance of *Bacteroidetes* fell, although only halfway into the diet intervention study at which point it began to increase gradually until the end of the study in both cloned and non-cloned pigs, which was not anticipated. This was mainly observed in the non-cloned pigs and may show that *Bacteroidetes* somehow adapt to the environment in the gut that was caused by HF/HE diet. Studies in mice have revealed that a microbiome dominated by bacteria belonging to the phyla *Firmicutes* and *Bacteroidetes* consist of genes that encodes several enzymes involved in digestion of

indigestible polysaccharides (Turnbaugh et al., 2006). Here it could be argued that the increase in both *Firmicutes* and *Bacteroidetes* observed in this thesis would hypothetically point to co-colonization of some bacteria belonging to the two phyla that may be able to reinforce the state of obesity by providing their host with extra energy obtained from digestion of indigestible carbohydrates. In fact in one study it was shown that bacterial co-colonization would increase their efficiency in fermentation of polysaccahrides and lead to increased adiposity (Samuel and Gordon, 2006). Although, it could be possible that the microbial community adapts to diet over time and therefore the timeline studies performed in this study are important in understanding of the dynamics of the gut microbiota. Metagenomic approaches would have increase the understanding of bacteria in their complex communities and may have provided a better understanding of the interaction between diet, gut microbiota and the role of collective genome of bacteria in development of obesity.

7.1.3 Göttingen minipigs: diet-obesity-gut microbiota

In **Paper III** the gut microbiota of Göttingen minipigs was characterized by next generation sequencing and overall the results showed a gut microbiota in obese state that was different than the two other pig models of obesity studied in this thesis. The Göttingen minipigs unlike Ossabaw minipigs and L x Y pigs were fed a normal chow consisting of a little more protein and fat than the lean group and fed *ad libitum* (Table 4). Interestingly their microbiota was different in the obese state than the other two pig models. However, the results obtained were from cecal and colon microbiota. The significant differences at genus level between lean and obese cecal microbiota was observed mainly in Gram-negative bacteria (**Paper III**). Even at phyla level the obese Göttingen minipigs had a higher abundance of Gram-negative bacteria such as *Tenericutes* and *Veruccomicrobia* (**Paper III**) than the lean Göttingen minipigs. These are interesting findings as the LPS of Gram-negative bacteria are implicated in causing metabolic endotoxemia which eventually leads to metabolic syndrome. This is because LPS induces inflammatory responses through which many cytokines are released that play a role in insulin resistance. Even though insulin resistance was not observed in these pigs, it is still noteworthy that the state of obesity and excessive feeding affects the composition of the gut microbiota and that it is not only HFD that can affect the gut microbiota. According to the results obtained in this thesis, it is suggested that the state of diet-induced obesity causes changes in gut microbial community which may reinforce the state of obesity. It is therefore important to find the role of the bacteria present in the microbiota in digestion and gut permeability of obese Göttingen minipigs. Here the hypothesis that obesity causes changes in the gut microbiota of obese Göttingen minipigs, and that their gut microbiota displayed a specific profile dissimilar to that of their lean counter parts, regardless of diet, is confirmed.

7.1.4 Ossabaw minipigs: HFD-obesity-gut microbiota

In **Paper III** the gut microbiota of Ossabaw minipigs was characterized by next generation illumina sequencing. However the results were based on a low number of animals. Nevertheless, the obese Ossabaw minipigs displayed a distinct microbiota from the lean Ossabaw minipigs. Indeed, two genera were identified in the gut microbiota of obese Ossabaw minipigs that had significantly lower abundances, namely *Prevotella* and *Lactobacillus*. These results are opposite of what has been found in T2D humans and genetically diabetic mice (Serino et al., 2012b;Larsen et al., 2010). However, the results are in agreement with other reports in obese humans (Ley et al., 2006b). Lower abundances of *Lactobacillus* have been shown to be correlated with decreased transepithelial resistance which may eventually lead to low grade inflammation through increased level of LPS and eventually cause MetS as discussed in **Paper III**. Most of the investigations on obesity induced metabolic endotoxemia have been done in mice models which have fewer similarities to humans than pigs and therefore this needs further investigation in pig models.

Other genera that have particularly been connected to T2D, such as an increase in genus *Oscillibacter* in HF diet-induced mice (Lam et al., 2012), were also investigated in this study. However, this was not observed in Ossabaw minipigs. Here it is important to note the many differences between mice and pigs and therefore it can be argued that the results obtained here may be better related to humans than mice, although this needs further investigation.

It is important to understand the contribution of the microbial community to metabolism of nutrients and their role in host energy balance. Studies have shown that nutrients affect the composition of the microbiota which causes changes in the dominance of some bacterial divisions, such as *Bacteroidetes* and *Firmicutes* that produce SCFA from digestion of otherwise indigestible dietary compounds which in turn provide their host with extra energy (Jumpertz et al., 2011;Schwiertz et al., 2009). In a more recent study it was reported that the abundance of *Firmicutes* was positively correlated with calorie intake and that the gut microbiota had a significant impact on energy balance in a Zebrafish model (Semova et al., 2012). One of the interesting findings by Semova et al. (2012) was that the microbiota plays a crucial role in stimulation of fatty acids uptake from intestinal epithelium and formation of lipid droplets in liver. These findings further confirm not only type of diet (HF/HE or carbohydrate rich) but also the nutrient load and calorie intake play a vital role in development and maintenance of obesity.

7.1.5 The animal model suitable for human studies: *Firmicutes-Bacteroidetes* dogma

In order to evaluate if an animal model is suitable for human studies in a niche such as gut microbiota studies, it is important to investigate the composition of the gut microbiota in the unaffected animal e. g. lean animals and the effect of a treatment on the animal. Here it will be discussed and argued which pig model have a gut microbiota in lean state that resembles that of humans. In **Paper II** the lean cloned

and non-cloned pigs gut microbiota was investigated. It was hypothesised that specific groups of bacteria in the gut microbiota bloom in response to either the state of obesity or HF/HE diet or both. In **Paper II** we confirmed that diet changes the gut microbial community in response to HF/HE diet and obesity. The lean cloned and non-cloned pigs that received HF/HE diet in restricted amounts, displayed a gut microbiota with high ratio of *Firmicutes* to *Bacteroidetes* despite being fed restrictively. The lean pigs had therefore characteristics of the obesity related microbiota in mice and humans described in the literature (Ley et al., 2005;Ley et al., 2006b). However other studies have shown that the gut microbiota in mice which received HF/HE diet was changed in lean state as discussed in **Paper II** (Hildebrandt et al., 2009). The same was observed in the lean Göttingen minipigs' cecal and colon microbiota with higher abundances of *Firmicutes* and lower abundance of *Bacteroidetes* compared to obese minipigs, however the later was not statistically significant (**Paper III**). The diet cannot be argued as the causative factor that could explain why the lean Göttingen minipigs had higher abundances of *Firmicutes* than the obese, as there was no significant difference between the lean and obese minipigs' chow. The lean Ossabaw minipigs in contrast, had higher abundances of *Bacteroidetes* and lower abundances of *Firmicutes* in their microbiota from colon and terminal ileum (Figure 4 **Paper III**).These results have been observed in other obesity-gut microbiota studies in pigs (Guo et al., 2008b;Guo et al., 2008a). In humans the normal microbiota in lean individuals as compared to obese individuals in case of *Firmicutes*/*Bacteroidetes* is consistent with animal models, with higher abundance of *Bacteroidetes* and lower abundance of *Firmicutes*. While the reports in on the abundance of these two phyla are different in different studies (Ley et al., 2006b;Zhang et al., 2009;Nadal et al., 2008;Santacruz, 2009). Nonetheless, based on the studies in this thesis and other reports, the gut microbiota seems to react to alterations in nutrient load and may therefore modulate absorption of nutrients (Jumpertz et al., 2011). The conflicting reports just mentioned, make it necessary to investigate the gut microbiota at lower taxonomical levels. It must be kept in mind that the pig models used in this thesis had different genetic backgrounds, diets and age and the number of pigs in each group varied and therefore it is difficult to make a comparison.

7.1.6 Gut microbiota along the intestinal tract

It was hypothesized that the intestinal microbiota is affected by diet and obesity in terminal ileum and colon in L x Y pigs and Ossabaw pigs. However due to very low number of animals in the Ossabaw group the statistical power of the data is low with only two ileal samples in the lean group and four samples in the obese group. Still, based on these low numbers of samples, there were significantly higher abundances of *Bacteroidetes* in colon than in terminal ileum both in lean and obese Ossabaw pigs just like the observations made in L x Y pigs, (**Paper II**). The same was observed in genus *Prevotella*, belonging to the phyla *Bacteroidetes*. These results together suggest site specification for this group of bacteria.

Most of the significant differences in Göttingen minipigs were observed from the ceal microbiota as compared to colon microbiota. The cecal microbiota may provide a better profile of the microbial community, as most of the fermentation processes by bacteria has been detected in cecum (reviewed in (Neish, 2009)). The results obtained in this thesis therefore suggest that the cecal microbiota is a better site to study microbial community.

8 Conclusive remarks and future Perspectives

According to the results obtained in this thesis, the cloned pigs did not display a smaller inter-individual phenotypic variation as compared to the non-cloned pigs and therefore the cloned pig does not provide a more suitable animal model than conventional pig for gut-microbiota-obesity related studies. Additional investigation is needed to optimize the cloning of experimental animals which could eventually offer a more controlled experimental model. However as it is now, it is not recommended to use cloned pigs in intervention studies. The method is low in efficiency, takes a long time and is costly and therefore non-cloned pigs may provide a better animal model.

HF/HE diet caused alterations in the gut microbiota of cloned and non-cloned pigs both in presence and absence of obesity. In lean cloned and non-cloned pigs, the overall composition but not the diversity of the faecal microbiota differed between the two groups, however this was not observed in the obese group. Therefore it is suggested that either obesity or excessive feeding or both may have camouflaged the cloning effect that was otherwise observed in the lean cloned group and not the obese cloned group. The findings from the gut-microbial community of cloned and non-cloned pigs agree with the hypothesis that the diet-induced obesity is related to changes in the ratio of *Firmicutes* to *Bacteroidetes* and especially a bloom in the phyla *Firmicutes* during the course of obesity as observed in the obese L x Y pigs. From the time-line analysis of *Firmicutes* and *Bacteroidetes* in faecal microbiota of obese cloned and non-cloned pigs, there was a correlation between increase in weight and relative abundance of *Firmicutes*. However, stabilization was observed in phyla *Bacteroidetes* over time. Hereby, diet is suggested to be the contributing factor causing changes in the gut microbiota and not obesity per se.

The gut microbial community of obese Göttingen minipigs displayed a very different profile than their lean counterparts. In Göttingen minipigs, obesity was connected to a radical change in several Gram-negative bacteria groups at both phyla and genus level in their cecal microbiota.

The gut microbiota of obese Ossabaw minipigs displays the characteristic of the gut microbiota found in obese subjects and not T2D. However the data in Ossabaw minipigs is based on a small number of animals and a larger number of animals in each group would provide a better understanding of the Ossabaw minipig's gut microbial community. However here it is suggested that the Ossabaw minipigs provide a better model of obesity and its metabolic disorders, not only based on the gut microbial profile but also due to increased blood parameters such as cholesterol and triglycerides in response to HF/HE diet.

One of the limitations of the studies performed in this thesis is that the different breed of pigs received different feed and their microbiota was investigated at different sites along the GI tract. Furthermore, there was low number of animals in each study which makes it difficult to conclude based on these small numbers.

Based on different observations made in phyla level in gut microbiota of the three pig models used in this study it seems necessary to investigate the microbial community in their respective niche and at lower taxonomic levels such as genus and if possible on the species level, given that the characterisation of bacteria at phyla level may give superficial information.

Overall, it is concluded that diet is a defining factor in gut microbial ecology and obesity as shown by three different animal experiments.

In an ideal experimental set up it would be preferable to choose four treatment groups in the same breed of animals. This is to assess the effect of different diets not only based on fat percent in the diet but also to investigate how quantity of feed and excessive calories would affect the gut microbial community. This could be done by giving each group one of the following diets so that together there would be four treatment groups: group 1; HF/HE diet fed *ad libitum*, Group 2; HF/HE diet in restricted quantities, Group 3; normal diet *ad libitum* and group 4; normal diet with restricted access to feed. Furthermore, it is suggested to investigate the microbiota in cecum and colon which according to this study provide a better profile of the microbial community. This can ideally be combined with measurements of intestinal permeability and blood parameters such as glucose, cholesterol, triglycerides and insulin. Using this set up, a correlation between gut microbiota, diet and metabolic syndrome can be made which would perhaps identify the animal model as suitable for such intervention studies.

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10 Appendix

10.1 Design of Probes by Arb

The sequence database and phylogeny software used here was Arb which runs on UNIX platform or LINUX. The software at the time of this study was freely available from http://www.arb-home.de/. Probes were evaluated by local alignment search tools such as BLAST on NCBI, Ribosomal database, Silva and Autodimer. The probes were not checked in different literature prior to design. The objective here was to select an oligonucleotide sequence complementary or specific to the region of target sequence which was 16S rRNA gene of bacteria belonging to genus *Prevotella*. The Arb probe design software was used in this case. In the Arb database bacteria hierarchy was chosen first. In the following steps the phylum *Bacteroidetes* was chosen, followed by the class *Bacteroidetes* and finally the whole genus of *Prevotella*. In Arb, in the program "probe design" a minimum amount of bases (15 bases) and the melting temperature Tm (45°C) of the probe was chosen and the probes were automatically designed by the software.

The probes' specificity was confirmed by checking them against all available 16S rRNA gene sequences for the named species in databases available such as Autodimer, silva, Ribosomal databse and BLAST in NCBI. NCBI Blast search was performed to check if the specificity of the probe.Furthermore the probes were tested for match in ribosomal database which matched the sequences found. From Silva the probe was checked to match sequences of the bacteria species by choosing probe base followed by probe check. In Autodimer the probe sequences were downloaded in FASTA format. The results obtained from aoutodimer are in the Table 8.

Table 8 Autodimer probe sequences

Name	Sequence	Length	Tm (°C)	Ex @ 260nm (cm^-1*M^-1)	Mass (g/mol)	fGC	°C) (kcal/mol)
Prevotella 1	ACGUUGAGCGUCUAC	15	20.2	47200	3351.3	0.53	-5.8
Prevotella 2	GACGUUGAGCGUCUA	15	19.5	51200	3391.3	0.53	-5.7
Prevotella 3	CAGACGUUGAGCGUC	15	33.7	77900	3680.5	0.60	-8.5
Prevotella 4	UGCAGACGUUGAGCG	15	45.4	88100	3720.5	0.60	-11.7
Prevotella 5	GCAGACGUUGAGCGU	15	45.4	88100	3720.5	0.60	-11.7
Prevotella 6	CGUUGAGCGUCUACA	15	20.2	47200	3351.3	0.53	-5.9
Prevotella 7	UUCCGCCCACUUUGU	15	33.0	39000	2644.8	0.53	-8.6
Prevotella 8	AUUCCGCCCACUUUG	15	23.0	50500	2958.0	0.53	-6.6

Arb is a very effective software for designing probes, however when using other databases such as NCBI and Ribosomal database to design probes, it is important to note that due to the huge amount of

information in these databases, the results are somehow inaccurate and difficult to achieve. Therefore these databases are probably more useful in case of verifying the probes for the chosen species to find if the sequences match the species.

10.2 Fluorescence in situ hybridization (FISH)

FISH was performed to visualize the two bacteria phyla, *Bacteroidetes* and *Firmicutes* in both cloned and non-cloned pigs (controls) in cross-sections of terminal ileum and colon (with content). The tissue samples were stored in formaldehyde, paraffin-embedded and three µm cross sections of colon and terminal ileum were prepared on SuperFrost® plus slides (Menzel-Gläser, Germany). A Cy3 labelled probe targeting the 16S rRNA of the Domain bacteria (5'- GCTGCCTCCCGTAGGAGT- 3') (Eurofins MWG Operon, Ebersberg, Germany) was used in order to observe distribution of bacteria in the lumen of the intestine (Trebesius et al., 2000). The bacteria were visualized in each intestinal section *in situ* by scanning (Axon GenePix®Autoloader 4200AL, Microarray scanner) and were evaluated (GenePix® Pro 6.0 Acquisition & Analysis software) and chosen for microscopy. A FISH was then performed on a cross-section of colon and terminal ileum from the selected slides using specific probes for phylum *Firmicutes* labelled with Cy3 (LGC354B 5'-CGGAAGATTCCCTACTGC-3' and LGC354C 5'-CCGAAGATTCCCTACTGC-3'). Another probe for identification of *Bacteroides* species CFB719 (5'-AGCTGCCTT CGCAATCGG-3') (Meier et al., 1999) (Weller et al., 2000) was used separately on the cross-section of colon and terminal ileum.

10.2.1 Results

The bacteria in the lumen of colon and terminal ileum were visualized *in situ*, where some of the tissue seemed to be partially degraded due to sampling mistakes, which in turn made it difficult to distinguish the bacteria in the lumen from the bacteria in partial degraded tissue. The bacteria from both phyla were identified, either among the disintegrated tissue debris or finely embedded in the mucosal layer as shown in the figure. We were able to detect the bacteria from both phyla *in situ*, showing an abundance of *Firmicutes* and *Bacteroidetes* both in the lumen of colon and terminal ileum while no difference was observed between the clones and controls.

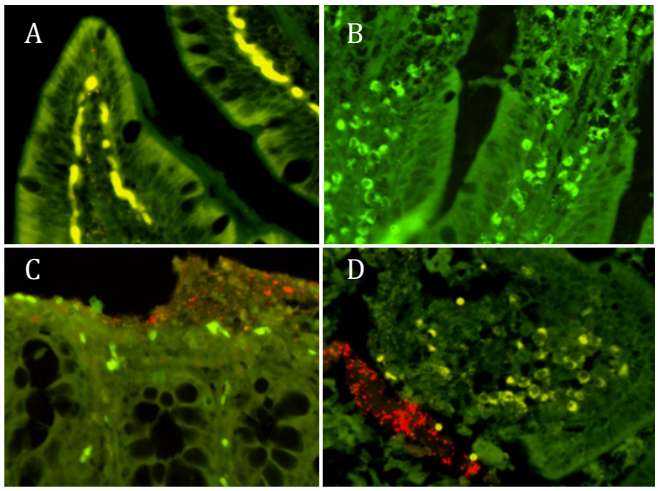

Figure 19 FISH of colon and terminal ileum cross-sections in three controls and one clone, the green colour is autofluorescenct tissue and red are the bacteria. A: FISH of *Firmicutes* on cross-section of terminal ileum (control), the tissue is intact and no bacteria are visible. B: FISH of *Bacteroidetes* in colon section (B), intestinal villi that is partially degraded. C: the red spots are bacteria belonging to the group *Bacteroidetes* in mucousal layer of colon close to the villi (clone). D: *Firmicutes* are visible close to partially degraded villi of terminal ileum (control).

10.3 Livak Method for relative quantification of bacterial fold-differences

Table 9 The steps towards calculation of Fold-changes by Livak method.

Sample		Gene
	Target	References
Callibrator	C_T(Firmi,calibrator)	C_T(GB-B,calibrator)
Test	C_T(Firmi, test)	C_T(GB-B, test)

Sample		Gene
	C_t firmi (Target)	C_tGB-B (References)
Control (Lean)	C_T(Firmi,Lean)	C_T(GB-B,Lean)
Test (Obese)	C_T(Firmi, Obese)	C_T(GB-B, Obese)

ΔCt (lean;Callibrator)=Ct(firmi, lean)-Ct(GB-B, lean)

ΔCt (lean;Callibrator)=15 – 16.5=-1.5

ΔCt (obese;test)=Ct(firmi, obese)-Ct(GB-B, obese)

ΔCt (Obese;Test)=12 – 15.9=-3.9

ΔCt (lean;Callibrator)=15 – 16.5=-1.5

ΔCt (Obese;Test)=12 – 15.9=-3.9

ΔCt=ΔC(test)-ΔCt(calibrator)

ΔCt=ΔC(obese)-ΔCt(lean)

=-3.9 – (-1.5)= -2.4

ΔΔCt=-2.4

$2^{-\Delta\Delta Ct}$=Normalized expression ratio

$2^{-(-2.4)}$=5.3

E.g. the obese have 5.3 fold more *Firmicutes* than lean.

10.4 Primers used in this study

Assay	Name	Primer 5'-3'	Study	
All Bacteria	Eub-8fm Eub-926r	AGAGTTTGATCMTGGCTCAG CCGTCAATTCCTTTRAGTTT	Paper I, II	(Mølbak et al., 2008)
All Bacteria	S-D-Bact-0907-a-S-20 S-D-Bact-1054-a-A-20	AAACTCAAAGGAATTGACGG ACGAGCTGACGACAGCCATG	Paper I, II	(Leser et al., 2002)
All Bacteria	804F 926R	GGATTAGATACCCNGGTAGTC CCGTCAATTCCTTTRAGTTT	Paper III	(in house design)
Bacteroidetes	798cfbF cfb967R	CRAACAGGATTAGATACCCT GGTAAGGTTCCTCGCGTAT	Paper I, II	(Bacchetti De Gregoris et al., 2011)
Firmicutes	928F-Firm 1040firmR	TGAAACTYAAAGGAATTGACG ACCATGCACCACCTGTC	Paper I, II	(Bacchetti De Gregoris et al., 2011)